Investigating the molecular mechanisms of curcumin against colorectal cancer stem-like cells

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

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September 2018

Abstract – Investigating the molecular mechanisms of curcumin against colorectal cancer stem-like cells

Background:

Curcumin inhibits the proliferation of cancer stem-like cells (CSCs) obtained from human colorectal cancer (CRC) tissues and adenomas. In NOD/SCID mice bearing xenografts from patient derived CRC CSCs, curcumin significantly decreased tumour growth and improved survival. Using an affinity pull-down assay with curcumin-coupled beads, curcumin pulls Nanog from cell lysate and directly binds the recombinant protein. In addition, individual Nanog domains were used to demonstrate binding of curcumin to the homeodomain (Nanog93-160). The transcription factor Nanog is crucial for the self-renewal of CSCs. Nanog expression in CRC tissue correlates with lymph node metastasis and poor prognosis. Since Nanog is not expressed in most tissues, including normal adult stem cells, it represents a therapeutic target specific to cancer cells. The aim of this thesis was to further characterise the curcumin-Nanog interaction.

Method:

Patient-derived tissues were collected and profiled for CSC markers including Nanog (n=90). The curcumin-Nanog interaction in the CSC population was investigated using Caco-2 cells and Nanog overexpressing cells ($HCT116^{GFP/Nanog}$) with matched control cell line ($HCT116^{GFP}$). Specifically, the effect of curcumin on transcriptional activity, protein expression and gene expression of Nanog and downstream targets (BMI1 and FAK) was assessed by luciferase reporter assay, western blot and RT-PCR. The effects of curcumin on CSC vs non-CSC populations (defined by aldehyde dehydrogenase) were also assessed using these methods. In addition, flow cytometry was used to assess $Nanog^{+}$ expression, proliferation and apoptosis. Subsequently, patient tissues were explanted and treated for 24 hours with curcumin. Explant tissues were then processed and analysed using flow cytometry (n=20). The effect of curcumin on $Nanog^{+}$ expression and proliferation was assessed.

Results:

Nanog expression was significantly higher in adenoma (6.85% n=6) and CRC tissues (3.47% n=46) compared to normal tissues (0.88% n=38). Curcumin significantly decreased the transcriptional activity of Nanog. In addition Nanog, BMI and FAK protein expression reduced following treatment. No change in Nanog gene expression was observed, although concurrent increases in BMI and FAK gene expression were detected. Further, a decrease in $Nanog^{+}$ and $Nanog^{+}Ki67^{+}$ expression was seen following curcumin treatment. A decrease in Nanog, BMI and FAK protein expression was detected following curcumin treatment in $ALDH^{high}$ cells. In patient explant tissues, no change was detected in $Nanog^{+}$ expression following curcumin treatment, however, there was a significant reduction in $Nanog^{+}Ki67^{+}$ expression.

Conclusion:

Data suggest $Nanog^{+}Ki67^{+}$ is targeted by curcumin in adenoma and CRC tissues. Ongoing studies to identify molecular and histological features that delineate responders from non-responders are underway. Nanog may serve as a biomarker in clinical trials to identify individuals most amenable to curcumin treatment alone or in combination for the prevention and/or treatment of CRC.

Acknowledgements

This one's for you Dad.

I would like to sincerely thank my supervisors, Karen and Anne for the unwavering support and guidance they have given me since moving to Leicester. I have had a tremendous time in the laboratory with everyone at the Leicester Cancer Research Centre (LCRC), particularly Ankur, Lynne, Emma and Jenny. Andy you brighten up every Tuesday. A special note of thanks to the generous patients who donated their tissues, Zahirah, Nalini and the Experimental Cancer Medicine Centres (ECMC) team who made the *ex vivo* explant work possible. Lastly, I am indebted to the team at Hope Against Cancer for teaching me to ride and sponsoring my PhD.

Abbreviations

ALDH	Aldehyde Dehydrogenase
ALK	Anaplastic lymphoma kinase
APC	*Adenomatous Polyposis Coli*
AMPK	AMP- activated protein kinase
BCA	Bicinchoninic acid assay
BMI	Body mass index
BRAF	*BRAF - Proto-oncogene serine/threonine protein kinase*
BSA	Bovine serum albumin
BUV	Bright ultra violet
CD	Cluster of differentiation
CDK	Cyclin D kinase
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability pathway
CMS	Consensus molecular subtypes

CNV	Copy number variation
COX	Cyclooxygenase
CRC	Colorectal cancer
CSC	Cancer stem-like cells
DCC	*Deleted in Colorectal Cancer*
DEAB	Diethylaminobenzaldehyde
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal growth factor receptor
EMEA	European Medicines Agency
EMT	Epithelial to mesenchymal transition
ESA	Epithelial surface antigen
FACS	Fluorescence-activated cell sorting
FAP	Familial Adenomatous Polyposis

FCS	Foetal calf serum
FDA	Food and Drug Administration
FIT	Faecal Immunochemical Test
FITC	Fluorescein isothiocyanate
gFOBt	Guaiac faecal occult blood test
HPV	Human papilloma virus
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IL	*Interleukin*
IMS	Industrial methylated spirit
KRAS	*Kirsten rat sarcoma 2 viral oncogene homologue*
LgR5	Leucine-rich repeat-containing G-protein coupled receptor 5
LIF	Leukaemia inhibitory factor
MACC1	Metastasis associated in colon cancer 1
MEM	Minimum essential medium
MLH	*Mut L homologue*

MSI	Microsatellite instability
MSH	*Mut s homologue*
mTor	Mammalian target of the rapamycin
NHS	Normal horse serum
NK	Natural killer
NSAIDs	Non-steroidal anti-inflammatory drugs
Oct4	Octomer binding transcription factor 4
OS	Overall survival
PBS	Phosphate buffered saline
PE	Phycoerythrin
PMS	*Post meiotic segregation*
PTEN	Phosphatase and tensin homolog
RPMI	Roswell Park Memorial Institute medium
SOX-2	Sex determining region Y-box 2
STAT	Signal transducer and activator of transcription

TNM	Tumour/node/metastasis
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor

List of publications and conference proceedings (selected)

Mahale, J., Singh, R., Howells, L.M., Britton, R.G., **Khan, S.M.,** Brown, K. Detection of plasma curcuminoids from dietary intake of turmeric-containing food in healthy human volunteers. Molecular Nutrition and Food Research, June 2018 DOI:10.1002/mnfr.201800267

Khan, S., Karmokar, A., Howells, L., Thomas, A.L., Bayliss, R., Gescher, A., Brown, K. Targeting cancer stem-like cells using dietary-derived agents – where are we now? Molecular Nutrition and Food Research, June 2016 DOI:10.1002/mnfr.201500887

Khans, S., Karmokar, A., Sidat, Z., Foreman, N., Moore, D., Higgins, J., Parrott, E., Theofanous, D., Hobbs. D., Howells, L., Thomas, A., Brown, K. Targeting Nanog in 3D explant models for the evaluation of cancer prevention agents. AACR conference proceedings, April 2018

Karmokar, A., **Khan, S.,** Howells, L., Norris, L., Cai, H., Britton, R., Singh, B., Aslam, M.I., West, K., McGregor, A., Bayliss, R., Winter, A., Steward, W., Gescher, A., Brown, K. Molecular mechanisms of curcumin and its in vivo efficacy in targeting colorectal cancer stem-like cells. Mutagenesis, July 2015 DOI:10.1093/mut

Oral Presentations (selected)

Khans, S., Howells, L., Thomas, A., Brown, K. Targeting cancer stem-like cells in colorectal cancer, **Abstract selected for oral presentation** at Royal Society of Medicine, London, March 2018

Khan, S., Burgess, S., Richards, M., Karmokar, A., Britton, R., Winter, A., Thomas, A.L., Bayliss, R., Brown. Exploring the interaction of curcumin with Nanog, **Abstract selected for oral presentation** M5 Structural Biology Conference, Leicester, September 2015

Khan, S., Thomas, A.L., Bayliss, R., Brown. Investigating Nanog as a potential druggable target in colorectal cancer stem-like cells, **Invited speaker** at Cancer Research UK Clinical Fellows Meeting, London, November 2014

Poster presentations (selected)

Khan, S., Karmokar, A., Sidat, Z., Foreman, N., Moore, D., Higgins, J., Parrott, E., Theofanous, D., Hobbs, D., Howells, L., Thomas, A.L., Brown, K. Targeting Nanog in colorectal tissue explant models for the evaluation of cancer prevention agents. DKFZ – Preventive oncology: Facing the challenges of cancer prevention and early detection, Germany, February 2018

Khan, S., Karmokar, A., Howells, L., Thomas, A.L., Brown. K. Title: Can combination therapy increase efficacy against colorectal cancer stem-like cells? BACR and ECMC meeting: Therapeutic interventions for cancer prevention, Bristol, July 2017

Khan, S., Karmokar, A., Higgins, J., Britton, R., Howells, L., Thomas, A.L., Brown, K. Curcumin as an agent for cancer prevention in colorectal cancer. UK/Dutch/Belgium Environmental Mutagen Society Conference, Leuven, Belgium, July 2017

Khans, S., Karmokar, A., Horner-Glister, E., Moore, D., Higgins, J., Howells, L., Thomas, A.L., Brown, K. Developing 3D platforms for the assessment of cancer prevention strategies in colorectal cancer. UK/Dutch/Belgium Environmental Mutagen Society Conference, Leuven, Belgium, July 2017

Khan, S., Karmokar, A., Britton, R., Winter, A., Thomas, A.L., Bayliss, R., Brown. Investigating Nanog as a therapeutic target in cancer stem-like cells. Accelerating Cancer Drug Discovery Through Structural Biology, Leicester, January 2016

Khan, S., Karmokar, A., Britton, R., Winter, A., Thomas, A.L., Bayliss, R., Brown. Investigating Nanog as a potential therapeutic target in cancer stem-like cells. National Cancer Research Institute Cancer Conference, Liverpool, November 2015

Prizes

'Best Oral Presentation Award' at UKEMS Conference, Oxford, September 2018

'Royal Society of Medicine Sylvia Lawler Oncology Prize' (Scientific section) at Royal Society of Medicine, London, February 2018

'Young Scientist Poster Award' at UK/Dutch/Belgium Environmental Mutagen Society Conference, Leuven, Belgium, July 2017

Grants

Cancer Research UK Research Bursary: £13, 175 awarded as principal applicant to support consumables during Academic Clinical Fellowship (April 2014- March 2015)

Hope Against Cancer Small Grants Programme: £24, 500 awarded as co-principal applicant to support purchase of microscope, camera, imaging software to aid biomarker and 3D organoids model development (July 2016)

Leicester Precision Medicine Institute – Precision Medicine project funding: £11, 500 awarded as co-applicant to support development of Nanog as a biomarker in colorectal cancer (Jan 2018)

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1.0 Chapter 1 - Background

1.1 *Colorectal cancer*

Colorectal cancer (CRC) is the fourth most common cancer in the UK. It accounts for 12% of all new cancer cases [1]. Over half of cases are diagnosed in those over the age of 70 years, with incidence increasing sharply after the age of 50 and highest in those aged 85-89 years [1]. Men are more likely to be diagnosed with CRC than women at a ratio of 13:10 [1]. CRC rates have increased by 5% over the last decade. This has been linked to a variety of reasons including effects of lifestyle and increased detection via the bowel cancer screening programme. Mortality rates have declined by 10-15% over the last decade, which may be explained by earlier detection and improved treatment options [2]. However, despite the introduction of the bowel cancer screening programme, over a quarter (26%) of patients are diagnosed with metastatic disease, with 5 year survival at 8% [2]. In addition even in the face of improved treatment options, approximately 50% of those treated with a curative intent will develop recurrent disease [1]. This results in CRC being the second leading cause of cancer deaths in the UK [1] and represents a significant burden on patients, families, healthcare providers and society as a whole.

1.2 Colorectal cancer risk factors

A number of risk factors have been associated with the development of CRC. These include hereditary and lifestyle of behavioural risk factors.

1.2.1.1 Hereditary risk factors

Inherited syndromes account for up to a quarter of CRC cases [3]. These include those with an identifiable mutation or syndrome and those where a high incidence of CRC is detected but no identifiable syndrome or mutation is apparent. Nearly 5% of CRCs are associated with a genetic syndrome such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) [3]. The remaining 20% of hereditary cancers are not associated with an identified genetic syndrome but individuals have a number of relatives (>3) who are affected by CRC [4]. In clinical guidelines, a hereditary link must be considered if a first degree relative who is less than 50 years of age is affected. In this case CRC risk is double and the risk of adenomatous polyps

increases by 75% [5]. If the relative is less than 45 years of age or more and one first degree relative is affected, then the risk is further increased [5] [6].

1.2.2 Environmental and lifestyle risk factors

A number of environmental and lifestyle factors have been associated with influencing the risk of CRC [7]. These are summarised in table 1-1. Approximately 54% of CRC cases diagnosed in the UK have been associated with preventable or modifiable risk factors [8]. These include intake of red meat and processed meat which is linked with an estimated 21% of bowel cancers [9]. In contrast, diets which are high in fibre may reduce the risk of CRC [10]. Moreover, dairy, calcium and garlic have been linked with protective effects against bowel cancer. However, the evidence here is not as strong as the links with processed meat [11]. In addition, the risk of CRC increases with weight gain particularly in men. Colon cancer risk is 18% higher in men who have a body mass index ranging from 25-29.9 kg/m^2 and approximately 50% in men who have a body mass index of greater than 30 kg/m^2 (obese) compared to those who have a normal weight (body mass index ranging 18.5-24.9 kg/m^2). In women colon cancer risk is 12% higher in those who are obese compared to those who are of a normal weight [12]. The risk may be higher in women who are premenopausal than postmenopausal [12]. Notably, adenoma risk is approximately 50% higher in obese individuals compared to those who have a normal weight [12] [13]. Aside from diet, alcohol intake [14] and cigarette smoking [15] are associated with an increase of 11% and 8% in CRC cases respectively. Physical activity is thought to be protective [16].

Table 1-1. Environmental and lifestyle risk factors for CRC

Type of Evidence	Increases Risk	Decreases Risk
Convincing evidence	Ethanol intake Smoking X-radiation/gamma radiation Processed meat High BMI	Physical activity
Probable evidence	Asbestosis Schistosoma Japonicum Red meat	Whole grains Dietary fibre Dairy products Calcium supplements Combined oral contraceptive pill

Adapted from International Agency for Research on Cancer and World Cancer Research fund [17]

1.3 Pathogenesis of CRC

Over 90% CRC cases are adenocarcinomas arising from adenomatous polyps (adenomas) with a smaller proportion arising from flat adenomas [18]. However, it is important to note that less than 10% of adenomas develop into CRC [18]. The reasons behind this are unclear. Studies have suggested indicators for progression to cancer include:

- Adenoma size (larger > smaller)
- Histology (villous > tubular)
- Dysplasia (severely dysplastic compared to non-dysplastic)

A pooled analysis of polyp data taken from the US screening programme and information on polyps suggested there were few identifiable risk factors between low risk non-advanced adenomas and advanced adenomas except HRT use in women and physical activity in men which reduced risk of adenomas [18]. A number of risk factors were investigated including alcohol intake and other dietary factors. Authors suggested that important risk factors which influence progression may not have been studied, or the classification of adenomas nullified any potential effect seen. Notably, inherited syndromes were not investigated in this population.

It is important to highlight that understanding the risk factors involved in the intermediate steps from adenoma to cancerous progression may lead to more targeted, less invasive colorectal cancer screening and targeted cancer prevention strategies, as well as giving a clearer picture of the process of carcinogenesis in CRC.

1.4 Multistep genetic model for CRC

There are two distinct pathways of CRC pathogenesis: the chromosomal instability pathway (CIN) [19] [20] and the microsatellite instability pathway (MSI) [21]. The majority of CRCs arise from the CIN pathway, which is characterized by defects in chromosomal segregation, telomere stability, and the DNA damage response system [19]. In particular the CIN pathway describes the adenoma to carcinoma sequence. Approximately 85% of tumours originating in the CIN pathway contain a mutation or inactivation of the *adenomatous polyposis coli (APC)* gene [22]. The chromosomal instability results in a multistep process from adenoma to carcinoma through the accumulation of mutations in oncogenes and tumour suppressor genes [19]. Vogelstein studied the frequency of mutations present in CRC at various stages. The model takes into account a number of genes that need to be mutated for cancer to occur, however the order in which they occur is not critical (see figure 1-1) [19]. This is important in cancer prevention as it highlights key genetic changes leading to carcinogenesis, which could be targeted or manipulated with cancer prevention agents.

The key mutations in oncogenes and tumour suppressor genes identified include:

- ***APC* gene mutation/loss (chromosome 5q21):** the *APC* gene is responsible for degradation of β-catenin [23]. Loss of function of *APC* tends to occur early in the sequence resulting in the expression of a short or non-functioning *APC* protein [24]. *APC* controls epithelial turnover through the wnt pathway and is important in the regulation of stem cell division. This is lost in 85% of sporadic cancers and is mutated in the germline of patients with FAP [23].
- ***Kirsten rat sarcoma 2 viral oncogene homologue (KRAS)* gene mutation loss (chromosome 12p12):** Inactivation of *KRAS* occurs in up to half of all CRCs [25]. A mutation or overexpression of KRAS leads to cellular proliferation and inhibition of apoptosis [26].
- ***Deleted in colorectal cancer (DCC)* allelic loss (chromosome 18q):** Deletion occurs in approximately 80% of CRCs and results in dysregulation of cell growth and apoptosis [19].
- ***p53* gene deletion (chromosome 17p):** Deletion tends to occur late and is detected in 1 in 3 CRC. p53 regulates the cell cycle and provides genomic stability [19].
- ***B-Raf proto-oncogene serine/threonine kinase (BRAF)* mutation:** BRAF mutations are present in approximately 10% of CRC. They are linked with serrated polyps and DNA methylation of CpG islands with a microsatellite instability phenotype. BRAF tumours are diagnosed at a later age, in a greater proportion of females, poorer differentiation, mucinous histology, microsatellite instability and larger primary tumours and poorer prognosis compared to other mutations [27].

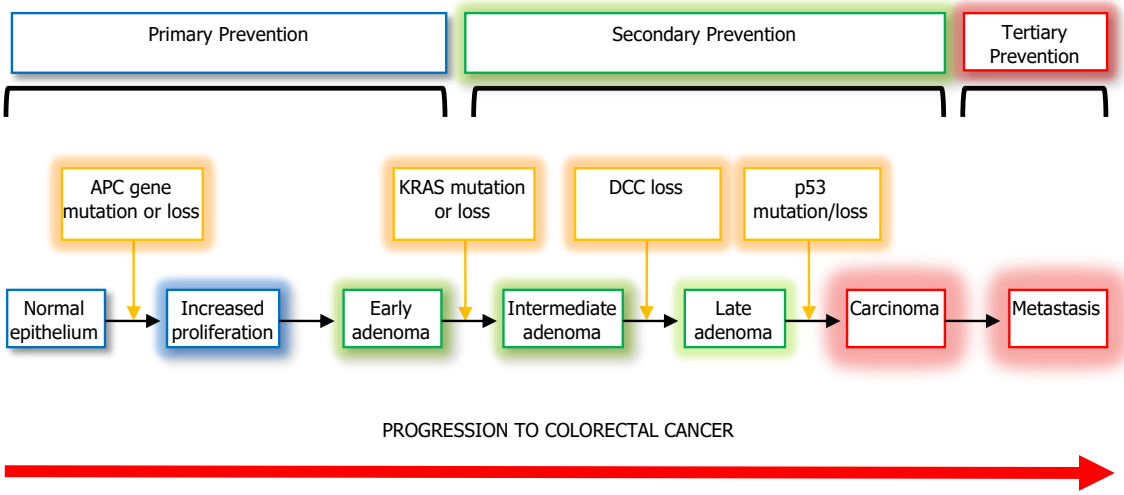


Figure 1-1. Outline of the multi-step genetic model for colorectal cancer highlighting the potential role of cancer prevention at various stages.

Abbreviations; APC - Adenomatous polyposis coli; KRAS - Kirsten rat sarcoma 2 viral oncogene homologue; DCC - Deleted in colorectal cancer

Microsatellite instability results from the loss of DNA mismatch repair (MMR) genes and is found in approximately 15% of all CRCs, 3% of which are associated with Lynch syndrome (inherited CRC syndrome) [28]. MMR genes include *mut s homologue (MSH) 1* and *2*, *mut L homologue (MLH) 1* and *post meiotic segregation (PMS) 1.* The most common mutations are found in *MSH2* and *MLH1* genes [28]. MMR results in loss of regulation of genes linked with proliferation and apoptosis e.g. apoptosis regulator bcl2 associated x protein. Tumours resulting from this pathway have improved prognosis compared to those arising from the CIN pathway, tend to arise in the proximal colon, have a lymphocytic infiltrate and are poorly differentiated with a mucinous or signet ring appearance [28]. In addition they gain no benefit from adjuvant fluorouracil based chemotherapy [29]. This highlights the link between understanding the pathogenesis of CRC and identifying a biomarker which can be used for prognosis and treatment for a defined population. This would also allow personalised prevention regimens to be established e.g. using MMR status as a means of selecting for particular treatment options.

1.5 Classification of CRC

Recently CRC has been classified into four distinct consensus molecular subtypes (CMSs) which are clinically significant in terms of the overall survival of patients with CRC (see figure 1-2) [30-33].

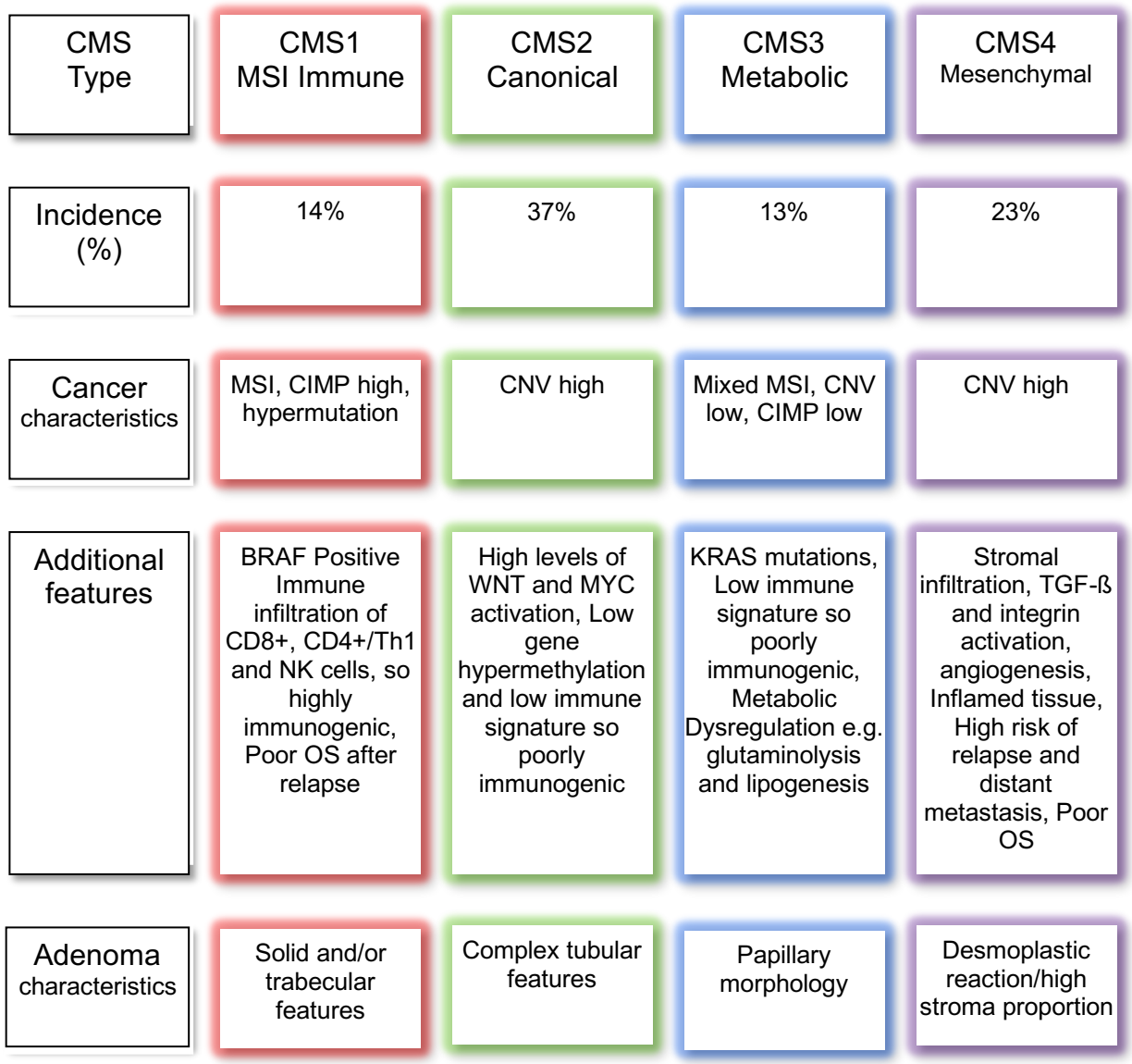


Figure 1-2. Sub-classification of colorectal cancer. The incidence and characteristic profile of each subtype is highlighted. In addition, adenoma characteristics are noted. These can be linked to patient outcomes, particularly in CMS1 and CMS4 subtypes. Some tumours could not be classified so the total is less than 100% [30-33].

Abbreviations; CMS – consensus molecular subtype, KRAS - Kirsten rat sarcoma 2 viral oncogene homologue, MSI – Microsatellite instability, CIMP – CpG island methylator phenotype, CNV - Copy number variation, BRAF - Proto-oncogene serine/threonine kinase, NK – Natural killer and OS – overall survival.

These were identified based on transcriptomic and gene expression profiling of thousands of patient samples by an international CRC subtyping consortium (CRC subtyping consortium). Specifically, the consortium assessed available gene expression data including MSI status, expression of common mutations, methylation status, copy number variation and immune status data to derive these subtypes. This will allow classification of pre-clinical models for screening initiatives and possibly help guide stratification in a clinical trial setting e.g. right and left sided cancers (see figure 1-3) [33-37]. However, despite these advances, more refinement of these categories is needed before they are able to guide personalised treatment of CRC in a clinical setting.

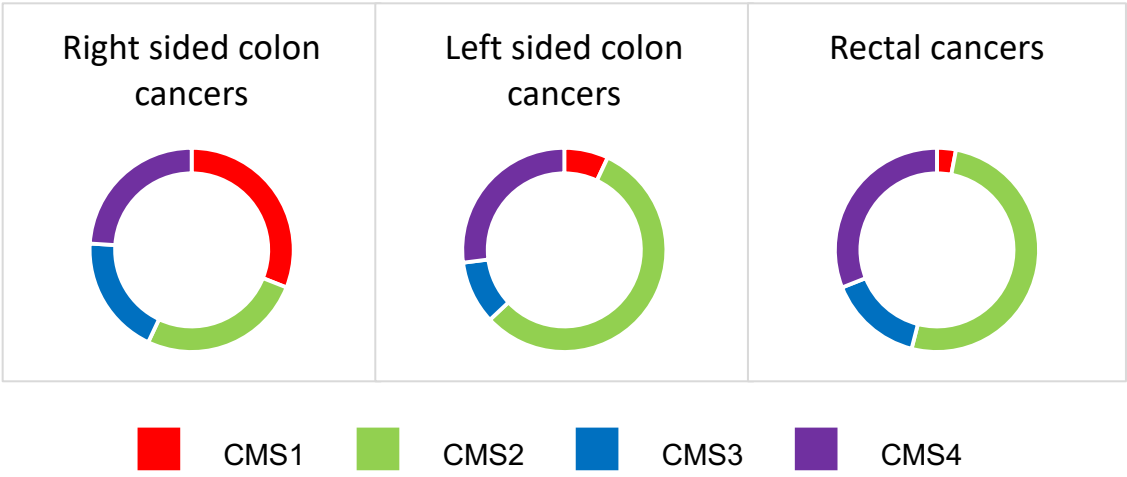


Figure 1-3. Consensus molecular subtypes of cancer and colorectal cancer site. This highlights the predominance of CMS1 (MSI and immunogenic subtype) in right sided cancers and CMS2 (canonical subtype) in left sided and rectal cancers. This may alter the management of colorectal malignancies in the future [38].

1.5.1 CMS1 summary

The first subtype of CRC, CMS1, originate from serrated polyps. They are mainly located in the proximal colon and have a predominance of BRAF positive tumours. In addition, they tend to be hypermethylated and have MSI. As well as this, they are highly immunogenic, with a predominance of lymphocytes in their microenvironment. The make up approximately 14% of sporadic cancers, and 3% hereditary cancers (mainly Lynch syndrome). In individuals where malignancy is detected early, a better prognosis is likely compared to those who have MSS disease. Due to the strong immune link,

immune check point blockade could be beneficial in this group of patients. Clinical trials are underway to assess this in advanced disease [30, 31, 33, 39].

1.5.2 CMS2 summary

These cancers tend to arise from the canonical-adenoma sequence described previously (figure 1-1). They are usually a differentiated epithelial cell type and demonstrate high levels of chromosomal instability as well as high somatic copy number alterations. In addition, they have a low mutation rate and have activated wnt, β-catenin and myc signal transduction pathways. These are mainly left sided lesions [31, 33, 39].

1.5.3 CMS3 summary

CMS3 is characterised as a metabolic subtype. It has genomic features which are consistent with chromosomal instability but in contrast to CSM2/4, it has a relatively low number of somatic copy number alterations. In addition, this subgroup has approximately 30% of tumours which are considered to be hypermutated (less than CMS1 but higher than CMS2/4). Notably, KRAS mutants are prevalent in this subtype (68%) [31]. Finally, this subtype appears to be most pathologically similar to normal tissue at gene expression level. In terms of associated precursor lesions, these include tubular adenomas with serrated features which is a mixed histopathology of CMSS1 and CMS2. Pathway analysis showed mRNA pathways were enriched for glutamine, fatty acid and phospholipid metabolism [30, 31].

In a subgroup analysis, tumours which were CMS3 and KRAS mutant (codon 12) with a distal location were associated with a shorter time to recurrence and poor prognosis. In addition, these tumours appeared to be poor responders to EGFR therapy [33].

1.5.4 CMS4 summary

Precursor lesions have a genetic signature consistent with the serrated adenoma pathway. They have low levels of hypermutation, MSS, and a very high somatic copy number. In addition, they have a mesenchymal phenotype with gene signatures consistent with an activated stroma, angiogenesis and integrin binding to matrix proteins and TGFB signalling characteristic of carcinoma associated fibroblasts. This is on a background of an inflammatory microenvironment. This proinflammatory environment

recruits T regulatory cells, T helper 17 cells and tumour promoting macrophages [33]. Also, IL 23/ 17 is present which is associated with colitis associated CRC where p53 occurs early on the transformation to dysplasia [30]. This is distinct from CMS2 where it occurs late [33]. Chemotherapy at advanced stages has minimal benefit, and resistance to EFGR therapy independent of KRAS mutation status is seen [31]. This demonstrates how subtyping can help guide prognosis and potential treatment strategy.

1.6 Staging of CRC

Following diagnosis of CRC, treatment is guided by cancer stage. There are 2 main classifications which are used world-wide. These are the tumour/node/metastasis (TNM) staging system and the Dukes' classification system (see table 1-2) [40, 41]. In addition, the stage at which a cancer is diagnosed strongly correlates with prognosis (see table 1-3) [42].

In the TNM staging system, the tumour is graded by its invasion through the bowel wall (T1-4), the lymph node component is assessed through the level of lymph nodes involved (N0-2) and the metastatic part is used to describe the presence of metastasis or not (M0 or M1) [40]. This can also be considered as stages 1-4, with 1 representing early disease and 4 metastatic disease. TNM staging is updated regularly. Dukes' classification is based on similar principles, but ranges from stage A-D where stage A represents early disease and stage D represents metastatic disease [41].

Table 1-2. Outline of TNM staging and Dukes' classification.

Stage	T	N	M	Dukes
0 (Carcinoma *in situ*) The tumour does not extend beyond the mucosa.	T*is*	N0	M0	–
1 The tumour invades into the submucosa (T1) or into the muscularis propria (T2).	T1	N0	M0	A
	T2	N0	M0	A
2a The tumour extends through the muscularis propria into the (T3).	T3	N0	M0	B
2b The tumour has infiltrated through the serosa (T4a). But there is no lymph node involvement (N0) or distant metastasis (M0).	T4a	N0	M0	B
2c The tumour has penetrated through the bowel wall and is attached to nearby tissues (T4b). There is no lymph node involvement (N0) or distant metastasis (M0).	T4b	N0	M0	B
3a T1 – T2 disease with metastasis to 1 to 3 regional lymph nodes (N1), or T1 disease with metastasis in 4 to 6 regional lymph nodes (N2a).	T1–T2	N1	M0	C
	T1	N2a	M0	C

3b T3 – T4a disease with metastasis to 1 to 3 regional lymph nodes (N1), or T2 – T3 disease with metastasis to 4 to 6 regional lymph nodes (N2a), or T1 – 2 disease with metastasis to 7 or more regional lymph nodes (N2b).	T3–T4a	N1	M0	C
	T2–T3	N2a	M0	C
	T1–T2	N2b	M0	C
3c T4a disease with metastasis to 4 to 6 regional lymph nodes, or T3 – T4a disease with metastasis to 7 or more regional lymph nodes, or T4b disease with any lymph node metastasis.	T4a	N2a	M0	C
	T3–T4a	N2b	M0	C
	T4b	N1–N2	M0	C
4a Any T, any N with metastasis to 1 distant organ or site e.g. liver, lung, ovary or non-regional lymph node (M1a).	Any T	Any N	M1a	-
4b Any T, any N with metastasis to more than one distant organ or the peritoneum (M1b).	Any T	Any N	M1b	-

Abbreviations – T – Tumour, N - node, M – metastasis, Dukes – Dukes' classification.

Adapted from [40].

Table 1-3. Prognosis of CRC based on Dukes' classification.

Dukes' stage at diagnosis	5 year prognosis (%)	
	Male	Female
A	95%	100%
B	80%	90%
C	65%	65%
D	5%	10%

Adapted from [41].

1.7 Management of CRC

The management of CRC can be divided into localised disease and metastatic disease [43].

1.7.1 Management of polyps and stage 1 colon cancer

In patients where a stage 1 cancer is diagnosed, the 5 year survival currently stands at >95%. This survival rate reflects the outcome when a segment of bowel and its associated mesentery is removed at surgical resection. For these patients, surgery is considered to be curative. Each patient case is discussed at the colorectal cancer multidisciplinary team meeting where pathological characteristics, imaging results and previous treatments are considered and an individual treatment plan made. Risk factors are also considered in an attempt to identify patients who are at high risk of recurrence [41].

In addition to stage 1 cancers, the number of polyps or adenomas detected are increasing following the introduction of the NHS bowel cancer screening programme.

Testing is offered to all individuals from age 60 to 74 on a two yearly basis. In 2017, the NHS bowel screening adopted the use of faecal immunochemical test (FIT), which will replace the guaiac faecal occult blood tests (gFOBt) over time [44]. The FIT allows specific measurement of human blood (rather than blood from a dietary source) in a more sensitive manner than gFOBt. In addition, it can be carried out with 1 sample [45]. This has led to more of the population engaging with screening with this test in pilot studies. Importantly, as it is more sensitive, it is able to detect a greater number of adenoma cases and has fewer false positives [45]. Following an abnormal result, an appointment with a specialist screening practitioner is arranged to discuss a colonoscopy or CT colonography [6, 45]. FIT can also be offered to symptomatic patients in specific situations e.g. where symptoms suggest CRC but the diagnosis is unlikely. In some health care systems a colonoscopy may be considered first line. In England, flexible sigmoidoscopy has been adopted, in addition to the FIT [45]. A single scope is offered to those aged 55-64 years of age. This confers a reduction in mortality and incidence when compared to on an invited basis only (43% vs 31% mortality and 33% vs 23% on incidence respectively). Overall, this results in approximately 3000 cancers being prevented [45].

Nearly all polyps are removed endoscopically. For many patients this is as effective as removal of a segment of bowel and associated mesentery with less surgical risk and recovery time. However, up to 20% of patients may have micro-metastasis at time of polyp removal [43]. There has been extensive work into identifying risk factors for recurrence e.g. the Haggitt classification for polyps with long stalks or the Kikuchi classification for sessile polyps, however these are not routinely used [46]. This is partly due to the difficulties of implementing the classification, heterogeneity of reporting and variable reproducibility. General associations with high risk of recurrence include poor differentiation of polyp, margin of normal tissue surrounding the polyp or adenoma is <1 mm, evidence of venous or lymphatic infiltration and evidence of tumour budding [47, 48].

The risk of developing CRC in individuals with adenomas has been classified into low, intermediate and high risk based on National Institute for Heath and Care Excellence (NICE) recommendation. This is used in England to help stratify patients and guide their treatment (see table 1-4) [49]. Based on individual risk, colonoscopy surveillance is offered between 1 and 5 years (see figure 1-4) [49]. Alternative options include a CT colonography for those who are unable to tolerate a colonoscopy or due to co-morbidities. If a CT colonography is not appropriate a soluble contrast enema can be

offered [40]. Though a soluble contrast enema may not be effective as detecting an abnormality as other modalities, larger polyps and CRC may still be detected.

Table 1-4. Identifying individuals at risk of CRC based on adenoma formation

Risk	Characteristics
Low	1-2 adenomas <10mm
Intermediate	2-4 adenomas <10mm OR 1-2 adenomas if 1 >10mm
High	5+ adenomas <10mm OR 3+ adenomas if 1 >10mm

Adapted from [49].

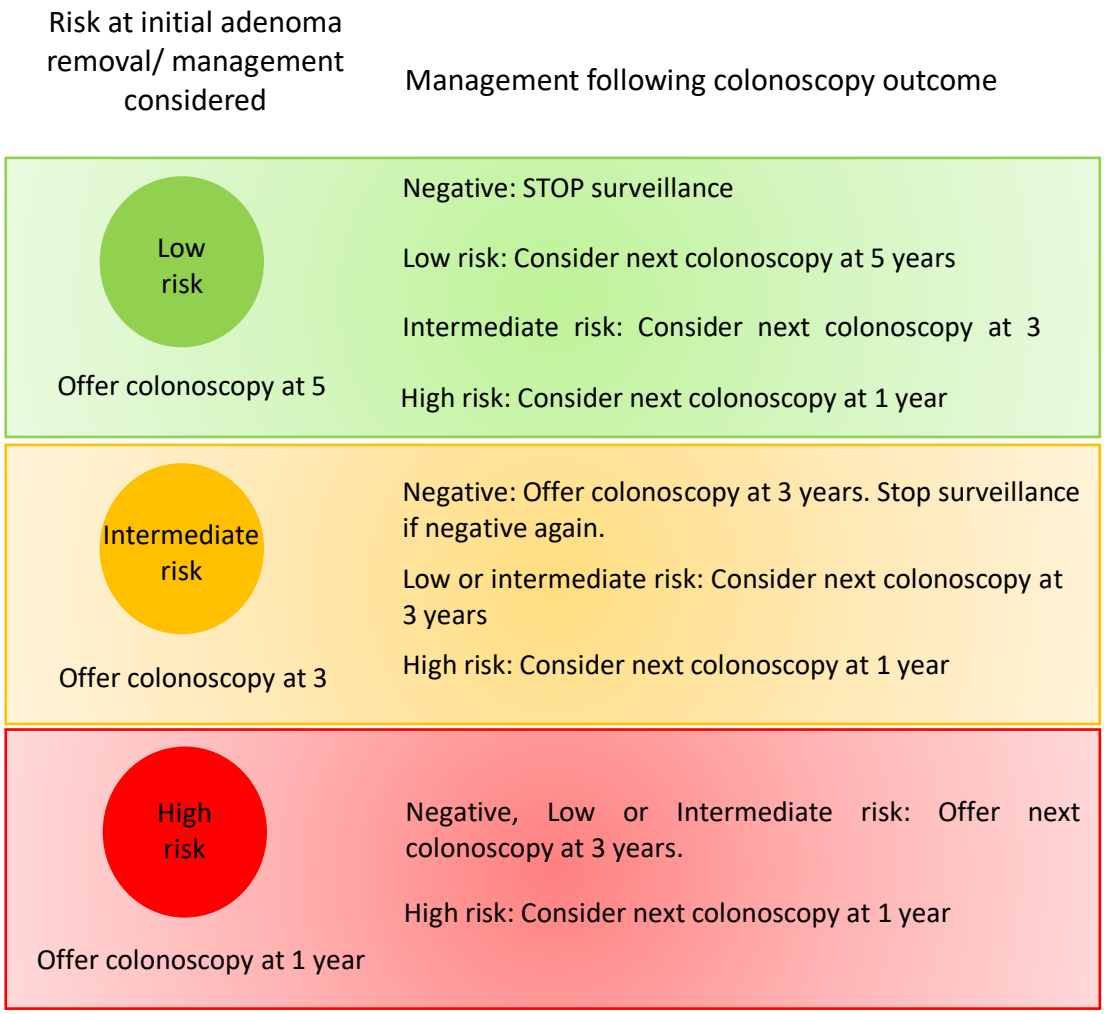


Figure 1-4. Management of individuals with adenomas. Upon removal of an adenoma, an individual is stratified into low, intermediate or high risk of CRC developing. This is based on Table 1-4. Based on this they are offered colonoscopy between one and five years. If an incomplete examination is performed, another may be carried out by a more experience endoscopist if required.

1.7.2 Management of stage 2 and 3 colorectal cancer

As with stage 1 disease, definitive treatment for stage 2 and stage 3 disease is surgery i.e. resection of a segment of bowel containing cancer and its associated mesentery and lymph nodes. Tumours are routinely removed laparoscopically if the lesion is suitable and expertise exists. Open and laparoscopic surgeries offer similar outcomes, however a shorter hospital stay and less morbidity is associated with laparoscopic surgeries [50]. Adjuvant chemotherapy is offered to those with high risk stage 2 and stage 3 disease. High risk stage 2 disease includes individuals where there is evidence of a poorly

differentiated tumour, lympho-vascular invasion, perineural invasion, obstruction or perforation of the tumour at presentation, a T4 tumour or less than 12 lymph nodes sampled for evidence of metastatic deposits [51, 52]. A combination of oxaliplatin with 5-fluorouracil and folinic acid or capecitabine is the treatment of choice (FOLFOX or XELOX), which reduces the risk of recurrence, and increases disease-free survival [53]. In stage 3 cancers it also improves overall survival of patients. In patients where FOLFOX may not be well tolerated or there are contraindications to treatment, single agent capecitabine may be used [53]. Over the last two years, the NHS has adopted molecular testing of mismatch repair proteins. This testing is part of the assessment for Lynch Syndrome, as well as the detection of sporadic CRCs associated with MSI [54]. Notably, sporadic cancers tend to be associated with epigenetic modification, which leads to the inactivation of the MLH1 gene. Specifically MSI-High tumours are associated with a distinct clinical and pathological pattern and better prognosis than MSI low or proficient tumours [55]. MSI deficient tumours are associated with resistance of 5-fluorouracil based chemotherapy and there is decreased overall survival in patients who have stage 2 cancers. Individuals who have stage 3 cancers continue to be offered chemotherapy [52].

Following surgery +/- adjuvant chemotherapy patients undergo a period of follow up. They are usually seen in clinic within four to six weeks of their surgery or chemotherapy and have a minimum of two CT scans within three years of diagnosis and a minimum of 6 monthly blood tests including serum carcinoembryonic antigen testing [53]. A surveillance colonoscopy is offered one year after diagnosis. If this is within normal parameters, then a follow up colonoscopy is offered at five years. Any further colonoscopies are at the discretion of the clinical team [53]. If there are any concerns, clinically, radiologically or biochemically of recurrence of disease, then re-investigation is started. Follow up can be stopped if this is the preference of the patient or where the risks of further tests do not confer added benefit [53].

1.7.3 Management of advanced colorectal cancer

Approximately 25% of patients present with metastatic disease. For half of these patients, metastatic disease presents in the liver. Surgical resection may be feasible for a minority of patients with liver or lung localised disease. This is available for approximately 10% of patients with liver metastasis [53]. Factors which preclude resection include number of deposits and their location, with assessment of remaining functional hepatic reserve, vascular involvement and evidence of extrahepatic disease.

Chemotherapy (FOLFOX or FOLFIRI in combination with cetuximab or panitumumab in patients with EGFR expression or RAS wildtype cancers for up to 16 weeks) prior to surgery improves progression free survival by 7-8% at three years in patients with resectable liver metastases and can help unresectable disease to be downsized.. Adjuvant chemotherapy (FOLFOX) can be given where chemotherapy before surgery is not used [56] [57]. The five year survival of patients with liver or lung resected metastatic disease is approximately 30% or 20-25% respectively. Subsequently trifluridine-tipiracil is also available. Raltitrexed can be used where 5-fluorouracil and folinic acid is not tolerated [57].

Most recently, there is emerging data regarding the efficacy of immunotherapy in MSI-H CRC management. Immunotherapy check point blockade often target either programme cell death protein 1 (PD-1) or anti-cytotoxic lymphocyte T lymphocyte associated protein 4 (CTLA-4) alone or in combination. Single agent, nivolumab [58] (PD-1 inhibitor) or a combination treatment using ipilimumab (anti-CTLA4 inhibitor) [59] are being investigated in CRC. Single agent and combination treatments have demonstrated objective response and improved quality of life indicators. These therapies are US Food and Drug Administration (FDA) but not yet European Medicines Agency (EMEA) licensed for previously treated metastatic colorectal cancer. Toxicities were present but these were manageable [60].

1.7.4 Management of rectal cancer

Total mesorectal excision is recommended for rectal tumours where patients are able to tolerate radical surgery. This reduces the local recurrence rate and improves survival [61, 62]. Preoperative chemoradiotherapy or short course radiotherapy is also recommended for rectal tumours. Preoperative chemoradiotherapy is useful for downsizing locally advanced tumours and is also more beneficial and less toxic than post-operative chemoradiotherapy [63].

1.8 Rationale for cancer prevention strategies in general

The treatment of advanced cancer is an area of intense research, often driven by the pharmaceutical industry however, the same attention is not given to cancer prevention strategies. The rationale of disease prevention has been realised in clinical practice. This is most notable in cardiovascular disease which can be used as a model [64]. In

this setting, the use of anti-hypertensive, anti-cholesterol and anti-platelet medication has led to a marked decline in cardiovascular-linked mortality and morbidity. Risk factors such as smoking, diabetes, high blood pressure and high cholesterol allow identification of individuals most at risk or individuals who are most likely to benefit from treatment. Furthermore, markers of efficacy, or biomarkers, can be identified and measured with relative ease e.g. blood pressure or cholesterol levels which have been linked with a reduction in morbidity and mortality. These can be considered as critical end points of disease prevention [65].

Therapeutic cancer prevention is the use of natural, synthetic or biological agents which aim to reverse, suppress or prevent cancer or delay the progression from premalignant cells to invasive disease [64]. Therapeutic cancer prevention can be classified in 3 ways; primary, secondary and tertiary. Primary therapeutic prevention aims to prevent the development of premalignant or cancerous cells in an otherwise healthy population. This population is at risk of developing cancerous lesions e.g. after exposure to a known carcinogen such as asbestosis or due to genetic-familial risk factors [65]. Secondary prevention aims to slow the progression of cancer. These individuals may have premalignant cancerous cells. This would include individuals who have adenomatous polyps or cervical dysplasia. Here therapeutic cancer prevention would aim to slow the progression to cancer to reverse premalignant changes [65]. Finally, tertiary prevention considers intervention following cancer therapy to delay or prevent the recurrence of cancer. In the context of CRC, primary prevention would be aimed at healthy individuals (young or old) who may or may not have a predisposition to colorectal cancer. Secondary prevention is aimed at individuals who have pre-malignant conditions e.g. adenomas and tertiary prevention would be given to those who have been treated for cancer (figure 1-1). In many respects the use of adjuvant chemotherapy can be considered as tertiary prevention therapy.

Disappointingly, therapeutic cancer prevention strategies have not yet realised the success of cardiovascular disease; this is partly down to the lack of validated surrogate biomarkers of cancer or other reliable predicators of efficacy. However, encouragingly a number of notable landmarks have been achieved. This includes the introduction of vaccination against human papilloma virus (HPV) 6, 11, 16 and 18 in an attempt to reduce the risk of cervical cancer [66] [67]. This has been achieved via the national immunisation programme introduced in 2008, and currently involves 2 vaccinations offered to 12-13 year old girls [68]. This represents a strategy where all girls are offered the vaccination, after identification of a risk factor necessary to instigate cancer

progression in nearly all cervical cancers [69]. This is in addition to the cervical cancer screening programme which aims to identify early cancerous lesions or premalignant cervical changes but does not prevent the pathogenesis of cervical cancer. A second example includes the use of tamoxifen or raloxifene for postmenopausal women with a uterus and at high risk of breast cancer [70]. Any potential benefit is balanced with the risk of thromboembolic disease or endometrial cancer. This represents a strategy where a high risk population is identified and modulation of risk factors is achieved via an oral medication. Moreover, adjuvant therapy is established in cancer care and could be considered as tertiary prevention.

In addition to providing a novel strategy to reduce the morbidity and mortality associated with cancer, a strong scientific rationale underpins therapeutic cancer prevention. Emerging evidence suggests premalignant tissues and early cancerous cells contain fewer genetic alterations compared to metastatic malignancies and exhibit less tumour heterogeneity [71]. This makes them favourable targets within a primary and secondary therapeutic prevention setting. Interestingly, high tumour heterogeneity has been suggested as a potential reason for seeing a limited impact on patient survival of targeted therapies for treatment of metastatic disease [71]. Besides, targeted therapies are limited to a small proportion of patients and toxicities are prevalent. The development of resistance as result of genetic and epigenetic changes means that maintaining drug efficacy is challenging for many patient cohorts. However, for most patients, the earlier a cancer is diagnosed and treated, the more favourable and durable the resulting outcome. This provides a strong rationale for treating individuals at high risk of cancer and those with known premalignant changes once the safety of agents has been established [72]. In fact, Vogelstein *et al* estimate a staggering 75% of all cancer deaths can be reduced by early detection and prevention [73].

1.9 Therapeutic prevention options in CRC

Epidemiological and clinical studies have highlighted a number of pharmacological and dietary agents with proven and potential anti-cancer activity against colorectal cancer. There is mounting clinical evidence for aspirin, NSAIDs including celecoxib and rofecoxib and metformin. There is also preclinical evidence for dietary agents including resveratrol and curcumin, whilst eicosapentaenoic acid has been shown to reduce polyp numbers in patients with FAP [74-76]. Only agents which have been tested in a sporadic CRC or an adenoma population will be reviewed here, as though the inherited cancers

have many similar pathological characteristics, the behaviour of agents in these populations may be different.

1.9.1 Pharmacological agents

1.9.1.1 Aspirin

A number of different pathways have been implicated in the proposed cancer prevention mechanism of aspirin. Pathways can be categorised as cyclooxygenase (COX) dependent or independent (see table 1-5) [77]. COX activity is mainly regulated by COX1 and COX2. COX1 activity is blocked via aspirin, resulting in an antiplatelet effect. This is important in malignancy where patients with cancer often have increased platelet activation which is associated with tumour metastasis. In addition, COX2 is also targeted, which is linked to an inflammatory state. In CRC tumours an increased expression of COX2 is detected, which is decreased following aspirin treatment. In addition inhibition of NF-κB via aspirin results in a reduction in proliferation, inflammation and angiogenesis [78].

Table 1-5. Outline of anti-cancer properties of aspirin.

COX independent mechanisms	COX dependent mechanisms
Inhibit IKK pathways leading to decreased inflammation NF-κb activation leading to apoptosis Inhibiting ERK signalling leading to decrease in proliferation and cell survival Activation of cytochrome C resulting in apoptosis	Increasing levels of ceramide leading to apoptosis Inhibiting angiogenesis Decreasing proinflammatory prostaglandins e.g. PGE2 often over-expressed in tumours Decrease PIK3CA signalling

In clinical studies, a daily aspirin reduced CRC incidence and mortality after long-term use (10-19 years). This is based on the meta-analysis of trials of aspirin for primary and secondary prevention of cardiovascular disease. Specifically, long-term risk is reduced by approximately 40% (HR 0.60, 95% CI 0.4-0.76). Daily doses from 75-1200mg were associated with a reduction in the 20 year risk of CRC death by 33% (HR 0.67, 95% CI 0.52-0.86) [79-84]. Data such as these, highlight the strong evidence for the anti-cancer activity of aspirin in CRC, which has led the US Preventive Services Task Force to recommend the use of aspirin in primary CRC prevention in individuals with cardiovascular risk factors.

The most common adverse events associated with long-term aspirin use are gastrointestinal bleeding and haemorrhagic stroke. Following daily or alternate day intake of low dose aspirin (<100mg), an additional ~14 deaths due to gastrointestinal bleeding deaths and three haemorrhagic strokes are detected per 1000 patients treated over a period of 10 years compared with two deaths due to gastrointestinal bleeding and < one death due to haemorrhagic strokes without [85]. Higher levels of these adverse effects are seen with older individuals (age >70 years). A careful balance needs to be struck, although the harm vs benefit ratio is often reported to be higher than it is [77, 86].

1.9.1.2 NSAIDs (including celecoxib and rofecoxib)

Selective COX2 inhibition via celecoxib [87, 88] and rofecoxib [89] have been studied due to a more favourable side effect profile than traditional NSAIDs. In individuals without a genetic condition but with a personal history of a polyp or an adenoma, two RCTs have demonstrated a decrease in polyp number. However studies did not have the appropriate follow up to be able to assess the effect on CRC incidence or mortality. The main adverse effects increased as a result of treatment were upper gastrointestinal bleeding (10%), chronic kidney disease and cardiovascular side effects (HR 4.61, 95% CI 1.50- 18.83) e.g. heart failure, stroke and myocardial infarction [90, 91]. This has limited the widespread use of selective COX2 inhibitors in colorectal cancer prevention. There are ongoing studies investigating lower doses of these agents in patients with metastatic disease e.g. celecoxib as an addition to standard chemotherapy FOLFOX in patients with metastatic colorectal cancer (NCT03645187).

1.9.1.3 Metformin

A number of potential mechanisms have been considered as the primary targets of metformin. These include direct and indirect targets. A potential route is directly targeting mitochondria in precancerous and cancerous cells, specifically complex I of the electron transport chain. In addition, an indirect route includes a global reduction in insulin which leads to a reduction in cellular proliferation, specifically in individuals who have high levels of insulin. These mechanisms share a common pathway via AMP- activated protein kinase (AMPK), inhibiting of the mammalian target of the rapamycin (mTOR) pathway leading to apoptosis, cell cycle arrest and a reduction in proliferation [92-94]. Additional mechanisms are considered in figure 1-5.

Interestingly, Nanog expression in hepatocellular cancer cells was decreased following metformin treatment after a transient increase. This occurred via the JNK pathway. Interestingly poorly differentiated tumours were found to have elevated levels of phospho-JNK compared to differentiated tumours. This may imply these tumours may be more sensitive to metformin treatment. In addition metformin conferred sensitivity to chemotherapy (5-FU). Further co-expression of Nanog and Oct4 conferred not only CSC properties but also promoted EMT in hepatocellular cancers [95]. Similar observations were made using CSCs derived from osteosarcomas. Specifically here, metformin was able to decrease sphere forming ability, conferred sensitivity to doxorubicin treatment and decreased the expression of 3 key CSC regulators; Nanog, Oct4 and Sox2 via the AMPK and AKT pathway [96]. This was supported by a second group considering the effect of metformin on osteosarcomas. Here metformin treatment decreased proliferation of CSCs and reduced Nanog and Oct4 protein expression [97].

A recent randomised control trial comparing low dose metformin with placebo, demonstrated a reduction in polyp number in the metformin arm. Non-diabetic individuals were recruited, and no serious adverse events were recorded in either arm [98]. Notably long-term follow up results are not yet available.

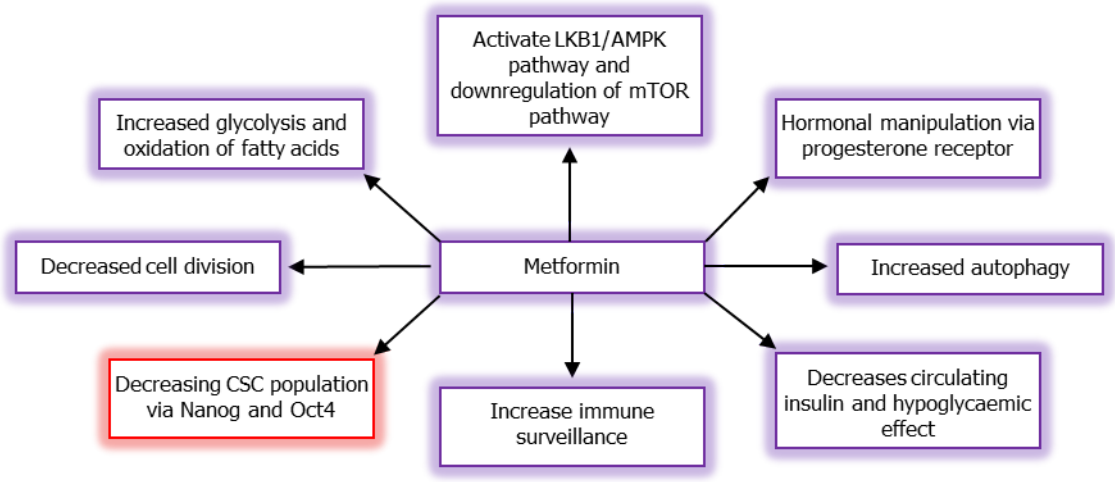


Figure 1-5. Potential mechanisms metformin is able to mediate anti-tumor activity. Those highlighted in red have been specifically implicated in targeting the CSC population.

Abbreviations; LKB1 - liver kinase B1; AMPK - AMP-activated protein kinase; CSC – Cancer stem-like cell; Oct4 - Octomer binding transcription factor 4 [99].

1.9.2 Curcumin

1.9.2.1 Background

Curcumin is a biologically active compound found in turmeric (*Curcuma longa*), a member of the ginger family and a rhizomatous plant. It is grown in south eastern tropical countries in Asia and is a commonly used Indian spice [100]. Epidemiological data suggest that a high intake of turmeric (~1.5 g daily per person) by Asian populations could be a contributory factor for a reduced incidence of inflammatory bowel disease (almost half) and colon cancer (one eighth) compared to western countries [101]. Its medicinal properties include treatment for wounds, inflammation, cardiovascular disease and antimicrobial properties.

1.9.2.2 Chemical Properties

Curcumin is a bright yellow polyphenolic compound. It is used as a food additive (E100) giving custard, cheese, butter and margarine their yellow colour. It is also used to dye clothing in the textiles industry. When the yellow powder is extracted from turmeric, it is approximately 75% curcumin with derivatives and curcuminoids (16% demethoxycurcumin, 8% bisdemethoxycurcumin and trace amounts of cyclocurcumin) [101].

Curcumin consists of two unsaturated carbonyl groups and two aromatic rings resulting in poor solubility (see figure 1-6). It is also photosensitive. These two properties can make it challenging to work with in a laboratory setting. In addition, the systemic bioavailability is low, with plasma concentrations of less than 0.14μM achieved after oral intake of curcuminoids at high doses up to 12 g/day in healthy individuals [49]. Curcumin levels peaked in plasma at 1-2 hours post intake. When 4 g and 8 g was consumed by patients with pre-malignant lesions the average peak curcumin levels in plasma were detected at 0.51 μM and 1.77 μM, respectively [40, 50]. At an oral dose of 2 g only subtle/trace levels of systemic curcumin were detected. This low systemic bioavailability could be attributed to poor absorption, poor solubility and rapid metabolism.

In addition, curcumin is more stable in acidic conditions than in alkali conditions e.g. in acidic conditions curcumin decomposes slowly with <20% degraded in 1 hour, whereas in alkali conditions all curcumin is degraded within 30 minutes. This makes it favourable in the gastrointestinal tract where the pH ranges from 1 in the stomach to 6 in the intestine. As a result, higher levels of curcumin are detected in the colon compared to systemic levels. In patients undergoing surgery for colorectal cancer, oral intake of curcumin (3.6 g/day) resulted in an average level of 12.7 and 7.7 nmol/g in normal and malignant tissue, respectively [52].

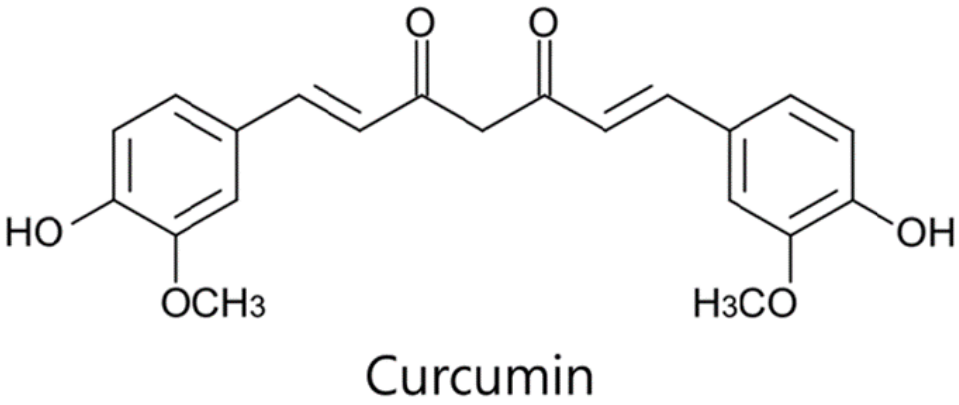


Figure 1-6.Chemical structure of curcumin. Curcumin is a polyphenol compound, with two aromatic rings and two unsaturated carbonyl groups resulting in poor solubility and bioavailability.

To determine the clinically achievable levels of curcumin in colorectal cancer tissues, a phase I study was carried out. This involved the administration of 2.35 g of oral daily curcuminoids for two weeks to patients undergoing colorectal endoscopy or resection of primary CRC disease. The non-specific binding capacity of curcumin was assessed via

washing colon mucosal tissue and measuring levels of curcumin found in cells. Levels of curcumin in washed samples were 20-75% less than those in unwashed samples, with mean tissue levels of curcumin reported as 0.05 μmol/g. Differences in curcumin levels were detected between right sided and left sided colon biopsies, but these changes were no longer detected once the tissues were washed potentially suggesting the concentration of cucrumin within colorectal cancer cells on the right or left side of the colon remains the same. It is also worth noting, though cells were thoroughly washed (upto seven washes so that the eluate had undetectable levels of curcumin), the amount in colonic cells is still likely to be less than this. In these patients plasma levels were detected (mean levels 0.014μM) and in urine (mean levels 0.08μM) [102].

Toxicities are related to dose and include bloating, reflux and loose stools [40]. An early phase clinical study with curcumin has demonstrated that curcumin is well tolerated in humans, up to a dose of 8 g/day when taken for three months orally [52], which supports its use as a therapeutic prevention agent.

1.9.2.3 Antitumor pathways targets

Curcumin is able to target a number of different pathways relating to proliferation [103], angiogenesis [104, 105], inflammation [106], apoptosis [107], cell cycle inhibition [108], reactive oxygen stress (ROS) [108] and metastasis [109] resulting in decreased carcinogenesis to varying degrees in a number of difference cancers [101, 110-113]. Pathways that relate to the efficacy of curcumin will be considered where evidence is obtained from *in vivo* models, human tissues or clinical studies where possible [114]. These pathways are highlighted in figure 1-7. A multi-pathway targeting agent allows a broad range of activity against malignancies. This is helpful as many pathways are often dysregulated in malignancies. In addition, agents where a single pathway is targeted e.g. EGFR or ALK in lung cancer [115] or BRAF [116, 117] in melanoma often develop resistance resulting in cancer recurrence.

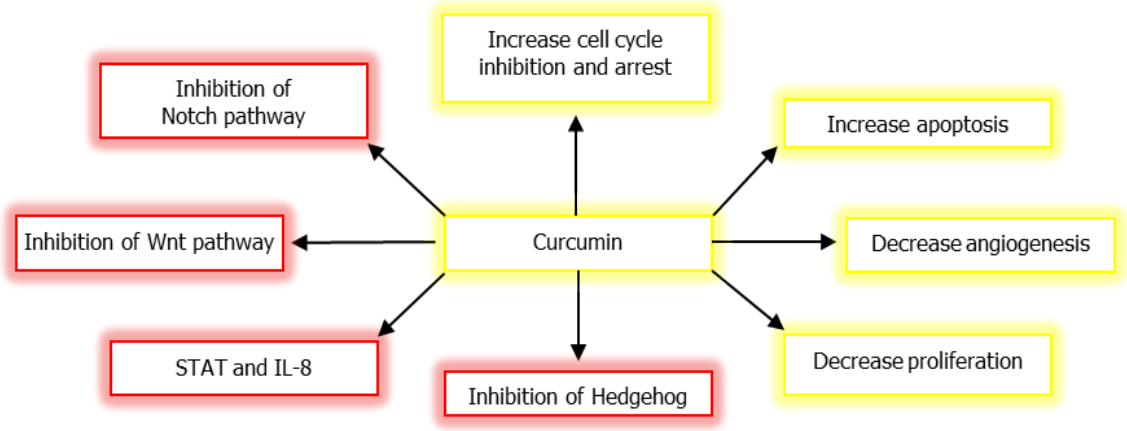


Figure 1-7. Potential mechanisms curcumin is able to mediate anti-tumor activity. Those highlighted in red have been specifically implicated in targeting the CSC population.

Abbreviations; STAT- Signal transducer and activator transcription; IL- interleukin [101]

Proliferation and inflammatory pathways:

Curcumin is able to modulate pathways associated with proliferation. In colorectal cancer this is primarily via inhibiting the wnt pathway, resulting in reduced expression of ß-Catenin via Nkd2, a negative feedback signalling pathway of wnt [118]. This is regulated via microRNA130a resulting in decreased proliferation of colorectal cancer cells following curcumin treatment. This study was carried out using xenografts produced from SW480 colorectal cancer cells and intraperitoneal injection of curcumin [118].

Curcumin treatment in numerous models leads to a reduction in NF-κB, [119] a decrease in expression of COX2 [120] and downregulation of cytokines e.g. TNF and interleukins/chemokines [121]. NF-κB is needed for cell growth, differentiation and proliferation thereby affecting a number of different pathways. It is often highly dysregulated in cancers [122]. However, despite numerous published *in vivo* and *in vitro* data suggesting an effect on proliferation and inflammation, following a Phase II clinical study of 41 subjects taking either 2 or 4 g curcumin for 30 days, no changes in proliferation or inflammatory pathway products were detected. Proliferation was assessed via Ki67 using immunohistochemistry. Inflammation was assessed via measuring prostaglandin E2 or 5-hydroxyeicosatetraenoic acid. This study used healthy mucosa, however in pre-malignant or malignant tissues it is possible that changes may have been detected. It is worth noting, a significant reduction in aberrant crypt foci

number in colon tissue (pre-cursor to malignant changes) was detected in participants taking 4 g curcumin [123].

Immune pathways:

Immune regulation is becoming a highly investigated area with the aim of developing cancer therapeutics. Curcumin has demonstrated efficacy in this area via inactivation of NF-κB leading to a decrease in interleukin 2 and T lymphocyte proliferation. In addition phagocytosis by macrophages is increased via a reduction in nitric oxide. Again, a phase 2 non-randomised clinical study investigating the effects of curcumin in patients with pancreatic cancer considered immune effects with varied results. A total of 24 patients took 8 g of curcumin/day for up to 18 months. Though the majority of patients did not have a response following treatment, 1 had a partial response, 1 had stable disease and 1 had growth in non-target lesions. In terms of immune effect, a number of cytokines were assessed including IL 6, IL 8 and IL 10 and IL 1 receptor agonist. Healthy individuals or patients baseline samples were used as comparators. In healthy individuals, these cytokines were undetectable. In patients, variable changes were detected following curcumin treatment e.g. patient with a significant response, had an increase in cytokine burden, whereas the patient with stable disease had a slight decrease over a period of months in cytokine burden [124]. The authors were not able to draw conclusions on cytokine response following treatment based on these results.

Cell cycle arrest and apoptosis:

Curcumin is able to downregulate cyclin D and thereby prevents cells entering the S phase of the cell cycle. In addition, blockage of cyclin D kinase 4/6 (CDK4/6) prevents cell cycle turnover and proliferation [125]. This has been a particularly effective method of cancer treatment in metastatic breast cancer e.g. Ribociclib is a CDK4/6 inhibitor recently licenced for first line treatment of metastatic disease [126]. In addition, curcumin blocks the action and expression of anti-apoptotic protein bcl2 and increases the activation of caspase 7 and 8 resulting in apoptosis. Also, curcumin increases p53 expression and p53-driven apoptosis [127]. These laboratory findings have also been observed in clinical studies. A phase 2 randomised study of 126 colorectal cancer patients taking either curcumin 360 mg three times a day or a placebo, demonstrated increased apoptosis as detected via a TUNEL assay compared to baseline samples in

patients taking curcumin. No change was detected in controls. There was also an increase in p53 and BAX and decrease in Bcl-2 [128].

1.10 Identifying and targeting cancer stem-like cells (CSCs) – standard CSC model

Advances in tumour biology support the 'cancer stem cell paradigm' [129]. Although there have been disputes over their existence, an expanding body of literature supports the hypothesis that a sub-population of cells are more tumorigenic in immunocompromised mice than others. These cells, termed cancer stem-like cells (CSCs) are thought to be responsible for driving the process of carcinogenesis and have demonstrated resistance against cytotoxic chemotherapies and radiotherapy, which may partly be due to their lower proliferative rate compared to non-CSCs [130]. Additionally, they are capable of unlimited self-renewal, similar to that observed in embryonic stem cells. CSCs have been implicated in disease recurrence [130] as they provide a reservoir of highly tumorigenic, chemoradiotherapy resistant cells to re-propagate a tumour following standard therapy regimens (see figure 1-8). It therefore follows that the CSC population provides a target against which cancer preventive or therapeutic agents can be developed. For example, a number of dietary and pharmacological agents may be able to target CSCs (see figure 1-5 and 1-6). CSCs are identified by a range of cell surface and intracellular markers, their ability to grow in immunocompromised mice plus their ability to form spheroids in non-adherent serum-free conditions. Side populations thought to correspond to CSCs can also be identified based on efflux of the Hoechst 33342 dye [131].

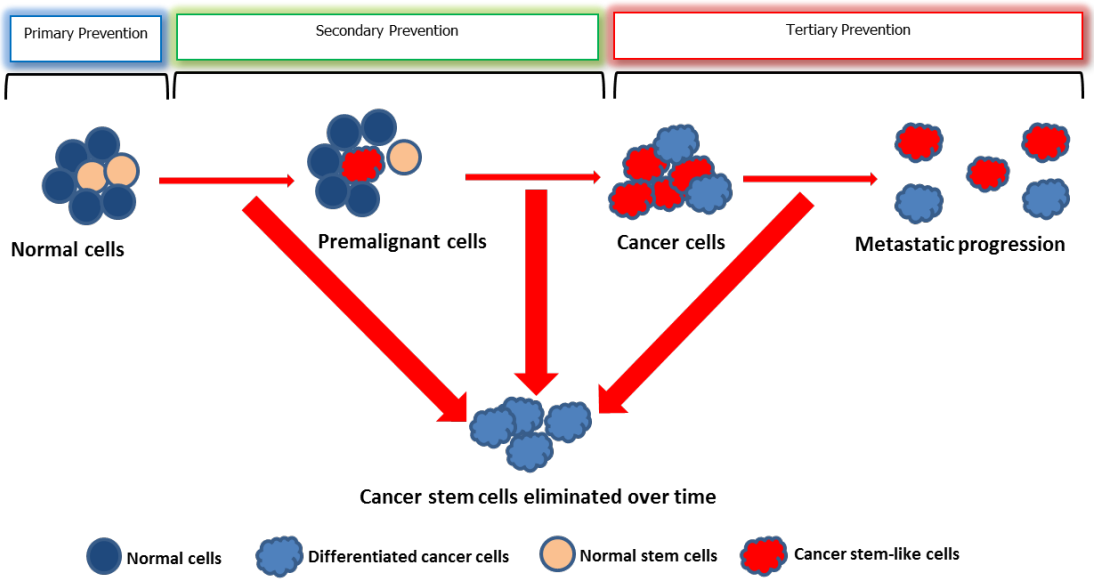


Figure 1-8.Therapeutic prevention strategies and CSCs hypothesis. Stem cells are critical to maintaining healthy tissues, allowing repair and replacement of cells to take place. In primary prevention, healthy normal cells can be targeted, to try and prevent early stages of carcinogenesis. If cells begin to show signs of pre-malignancy, they can be targeted with secondary prevention strategies Lastly, following cancer treatment, tertiary prevention can be used to reduce the risk of recurrence. It is critical to target CSCs, as left unchecked they can lead to tumour recurrence and resistance.

There are however, a number of challenges to consider. Though CSCs have been identified in a number of different cancer settings, their phenotype is variable compared to embryonic stem-cells, which have a fixed phenotype and are therefore more readily identifiable [131]. In addition, the proportion of CSCs within each cancer is also variable. For example, in melanoma, there are reports that the CSC population is as high as 1 in 4 of all cells, whereas in other tumour types such as breast or colorectal cancer, the proportion reported has been less than 5% [131]. Notably, even in the same tumour, different sub-populations of CSCs may be detected, with different roles in carcinogenesis [132]. For example, breast cancer cells with Aldehyde Dehydrogenase ($ALDH^{high}$) activity exhibit the same tumourigenic behaviour as cluster of differentiation (CD) $44^{high}/CD24^{low}$ cells when transplanted into immunocompromised mice. These sets of markers have been linked with discrete sub-populations of breast CSCs and different behaviours within a tumour [132]. Breast CSCs expressing $CD44^{high}/CD24^{low}$ have been associated with a relatively quiescent behaviour compared to $ALDH^{high}$ CSCs and are located at the periphery of a tumour site. The authors of this study suggested that this was due to the $CD44^{high}/CD24^{low}$ phenotype being associated with tumour invasion. In contrast, the $ALDH^{high}$ cells were located centrally within a tumour, and associated with

proliferation. Therefore, cells presenting with either of these markers display different breast CSC phenotypes and may capture distinct characteristics within a tumour [132]. It is important to note, CSC markers are difficult to validate, for example cluster of differentiation CD44 has a role in cell adhesion and attachment, therefore, cells with $CD44^{high}$ expression may grow readily in immunocompromised mice for this reason and not due to a CSC phenotype [131].

Moreover, subcutaneous (*s.c*) injection into immunocompromised mice, does not fully recapitulate the tumour microenvironment, or account for the role of the immune system in tumourigenesis [131]. In addition, this model does not take into account cell to cell contact or the presence of an extracellular matrix. The limitation of this approach for identifying CSCs has partly been overcome using lineage tracing studies. In one such example, the authors developed a genetically engineered glioblastoma mouse model, which enabled fluorescent tagging of CSCs within a tumour population. This allowed observation of a slowly dividing fluorescent population that was resistant to standard chemotherapy for glioblastoma (temozolamide), and corresponded to the CSC population; however, the non-fluorescent tumour population (non-CSCs) was reduced with chemotherapy treatment. Thereafter, the fluorescent population underwent proliferation resulting in tumour recurrence [131]. Similar models have helped to demonstrate CSC resistance against many cytotoxic chemotherapies and radiotherapy, which may partly be due to their lower proliferative rate compared to non-CSCs. Due to this, CSCs have been implicated in disease recurrence as they provide a reservoir of highly tumorigenic, chemoradiotherapy resistant cells to re-propagate a tumour following standard therapy regimens [131]. It therefore follows that the CSC population provides a target against which cancer preventive or therapeutic agents can be developed. However, the simplicity of this approach is now being re-considered due to the plasticity of CSCs.

1.11 Plasticity of CSCs

Though the traditional model of CSCs was held for many years, more recent research has demonstrated that CSCs may be more plastic in nature, particularly in solid tumours compared to haematological malignancies where CSCs were first characterised [133-136].

The colon contains millions of crypts which allow it to function as the main organ absorbing water and nutrients from the gut. Each crypt contains approximately 2000 cells, with 3 distinct populations; stem cells, transit amplifying cells and differentiated cells (see figure 1-9). The bottom of the crypt contains the stem cells. These are able to self-renew, but also give rise to transit amplifying cells. These are able to amplify going up the crypt and then go on to give rise to differentiated cells. Three main groups of differentiated cells are seen in a colonic crypt: endocrine cells which are needed for hormonal control, goblet cell required for the secretion of mucus and enterocytes which are needed for the absorptive role of the gut. This regulation is controlled by a number of pathways ensuring healthy homeostasis of the colon, however the wnt pathway and bone morphogenic protein pathway are key in regulation [137-140]. Alternative pathways which are important to stem cell development and regulation include hedgehog and notch. Curcumin is able to modulate these pathways as demonstrated in figure 1-10 [133, 134, 141].

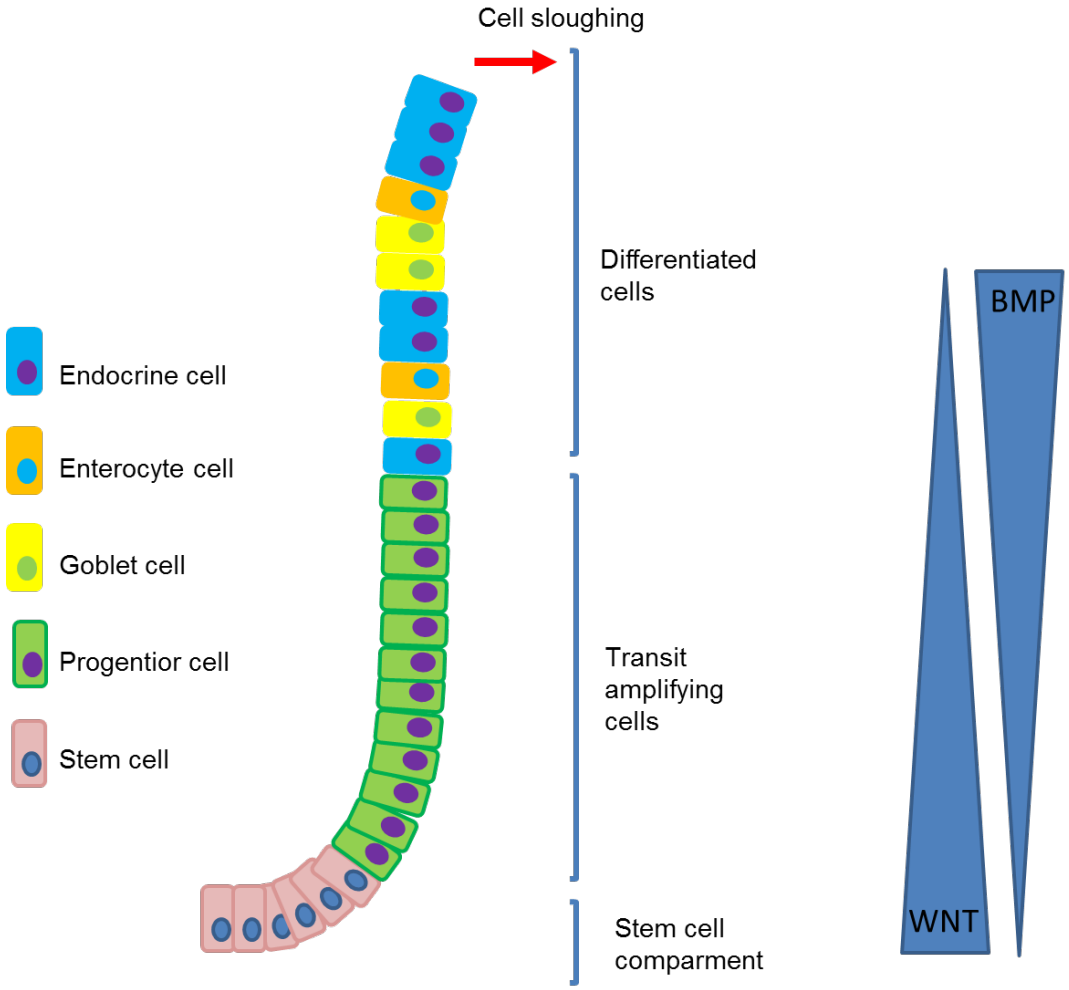


Figure 1-9. Features of a colonic crypt. Each crypt contains a stem cell compartment. These cells are able to self-renew and divide due to local stimulatory control. They can create new stem cells or transit amplifying cells. These grow up the crypt, dividing into differentiated cells. This process is controlled via the wnt and bone morphogenic pathway (BMP).

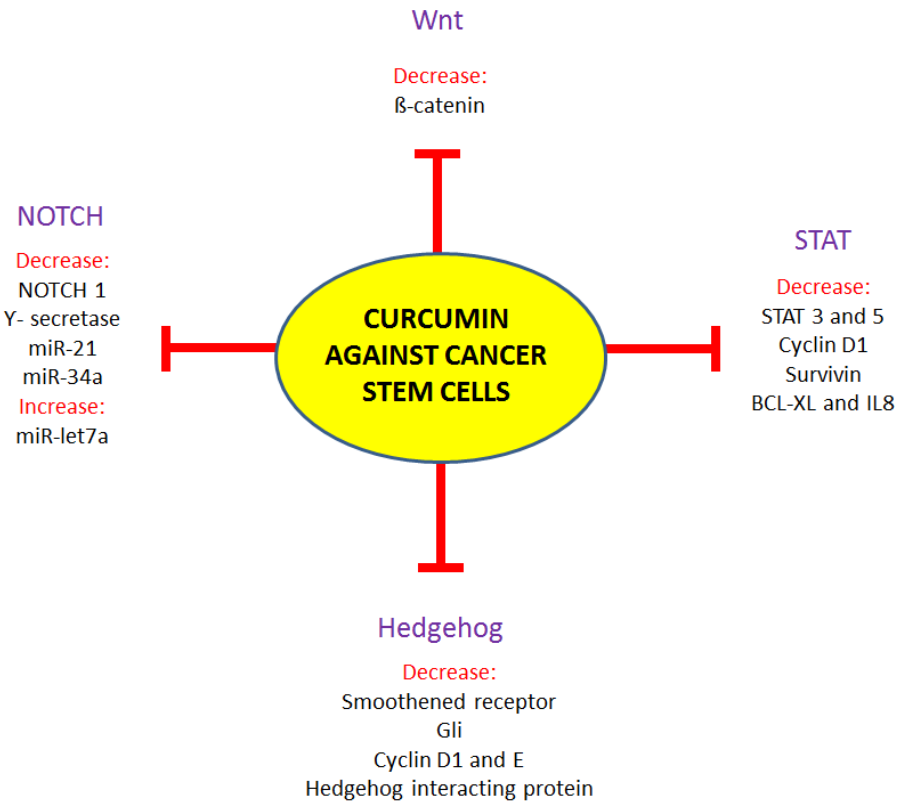


Figure 1-10. Curcumin and CSC regulation. Curcumin is able to modulate a number of pathways which are critical to cancer stem cell regulation.

When specifically considering intestinal cells, tumours can be induced following an APC deletion. This is a common deletion in CRCs. In an experimental model, these cells were labelled with a red dye allowing easy identification of mutant cells. Subsequently $LgR5^+$ cells were induced, these were then labelled blue (approximately 10%). Only some blue cells i.e. a fraction of $LgR5^+$Apc mutant cells were able to form early tumours and function as CSCs i.e. just by the nature of being $LgR5^+$ did not confer the ability to form tumours [142]. This challenges the early theories where stem cell niches are not very prevalent and do not divide continuously or quickly. It appears in some studies, stem cells can divide to form zero, one or two daughter cells. Crucially, depending on the context of CSCs, some differentiated cells can go on to replace stem cells. This is known as the neutral competition model [133].

This plastic nature has been confirmed following the ablation of $LgR5^+$ cells in the intestine. Here there is no loss in the integrity of epithelium or lack of function of the

intestine, instead early progenitor or enterocytes revert to form $LgR5^+$ cells. This is due to the environmental niche in the crypts i.e. activation of wnt, activation of EGFR, and protection of the TGFB and bone morphogenetic proteins allowing the characteristics and properties of cancer stem cells to be gained [12, 133, 143].

The connection between CSCs and epithelial to mesenchymal transition (EMT) is not fully characterised, however there is an indication that it may play an important role in metastasis of epithelial tumours, as these properties allow migration and invasion. It has been noted that there is an association of high levels of EMT transcription factors with CSC phenotype and these properties have been detected in metastatic deposits as well those cells initially forming the primary tumour. It is clear that some cells lose their EMT properties upon reaching their metastatic deposit site. The regulation of this is poorly understood and may depend on factors in the microenvironment or niche in this new area. In addition, it appears some cells are able to migrate or invade without activation of EMT pathways [133, 144]. Therefore, though there appears to be some overlap between CSC and EMT pathways in certain solid tumours and contexts, they are distinct pathways and appear to be regulated differently. This provides further evidence for the mobile nature of the CSC and non-CSC phenotype.

There have been attempts to try and target specific metabolic pathways which may be different between CSCs and non-CSCs, however it is likely the pathways which are utilised are very dependent on the environmental stimuli present to an individual cell and not related to stem cell status [133].

The new paradigm of CSCs therefore challenges the fundamental core beliefs held regarding CSCs particularly when applied to the intestine. For example non-cancer stem cells are not slow cycling or quiescent when considering the intestinal crypt, they make up 10% of the population and are cycling constantly. However, though these cells may be targeted by chemotherapy, progenitor cells or dormant differentiated cells in G0 of the cell cycle are spared. It may be that these cells are replaced in the intestinal crypt by cells which harbour characteristics of CSCs and therefore contribute to resistance and relapse [136, 144]. A number of pharmaceutical companies have developed drugs which target CSCs, however this approach may also damage healthy stem cells. In addition to the plastic nature of these cells, a targeted approach may prove futile particularly in a metastatic setting, where phase 1 studies are often trialled. Therefore,

agents which target a CSC and non-CSC population may be needed. This of course needs to be balanced with side effect profiles to achieve full potential in a clinical setting.

1.12 Importance of ALDH in CSCs and usefulness as a CSC biomarker

ALDH is a detoxifying enzyme and is involved in the oxidation of aldehydes to carboxylic acids. A number of ALDH subsets e.g. ALDH1, ALDH3 and ALDH8 are able to detoxify reactive aldehydes which are products of lipid peroxidation [145]. Others are involved in the retinoic acid pathway and catalyse conversation of retinaldehyde to retinoic acid. These subsets (ALDH1A1, ALDH1A2 and ALDH1A1) are key mediators of CSC expansion and differentiation. Moreover, ALDH has been associated with inactivating alkylating agents e.g. cyclophosphamide resulting in therapeutic resistance. In addition, cells which have a relatively high expression of ALDH have been implicated in having a mechanistic role in metastasis [145]. Over 19 different ALDH functional genes and numerous splice variants have been characterised resulting in a number of different ALDH isoenzymes. Notably, the nomenclature of ALDH enzymes has changed over time e.g. ALDH1 could relate to ALDH1A1, ALDH1A2, ALDH1A3 etc and some terms are used interchangeably e.g. ALDH1A1 and ALDH1 or ALDH [145].

1.13 Nanog and regulation of CSCs

Pluripotency of ESCs is regulated by a network of three key transcription factors: octomer binding transcription factor 4 (Oct4), sex determine region Y-box 2 (Sox2), and Nanog [146] [147]. Though the transcription and expression of Nanog has been shown to be regulated by Oct4 and Sox2 heterodimers, Nanog is essential for stem-like properties and is independent of other factors e.g. leukaemia inhibitory factor (LIF) and the signal transducer and activator transcription 3 (STAT 3) pathway, placing Nanog at the top of the regulatory pathway for stem-like properties [148] [147]. The functional significance of Nanog in CSCs was recognised with the finding that shRNA mediated knock-down of Nanog inhibits tumour development, whilst overexpression is associated with pro-tumorigenicity and drug resistance [149] [150]. Nanog is exclusively expressed in pluripotent cells such as early embryonic cells and in germline stem cells [147]. Nanog levels reduce upon differentiation and so are not expressed in healthy adult cells therefore it represents a novel therapeutic target specific to cancer and premalignant cells [147]. In addition, CRC cells expressing Nanog, display characteristics of CSCs and epithelial- mesenchymal transition (EMT) via insulin growth factor/STAT3/Slug axis

[151]. This suggests modulation of Nanog may also be governed by the tumour microenvironment, and helps to understand the link between Nanog with tumour initiation and metastasis.

1.13.1 Functional significance of Nanog and Nanog domains

Nanog is a multi-domain protein consisting of 305 amino acids [152]. Nanog is composed of three domains; a serine-rich N-terminal domain (amino acids 1-96), a helical homeodomain (amino acids 96-155) and a C-terminal domain (amino acids 156-305). The C-terminal domain contains a W-repeat with an 8-fold repetition of a W-X-X-X motif (human) where W denotes tryptophan and X any amino acid [153] (see figure 1-11). The N-terminal region is not only crucial for the transcriptional activity of Nanog, it contains a nuclear localisation sequence and is regulated mainly through post translational modifications e.g. phosphorylation of serine, threonine and proline [153]. The homeodomain is required in DNA binding, with its DNA motif recently being determined [152]. Further the murine homeobox has been crystallised, and been shown to be helical in nature [152]. Additionally it has been studied using NMR (2KT0). Within the homeodomain is a nuclear export motif allowing the transcription factor to be transported in and out of the nucleus [154]. Interestingly, a short 70 amino acid sequence containing the homeodomain is able to induce Nanog properties in protein deficient somatic cells suggesting this region is critical in the regulatory role of Nanog [155]. The C-terminal region contains the transactivation domains. Importantly, Nanog forms dimers via the C-terminal domain. This is an important property required for interaction with proteins related to pluripotency, as the dimer, not the monomer is able to place Nanog at the top of the stemness hierarchy [153].

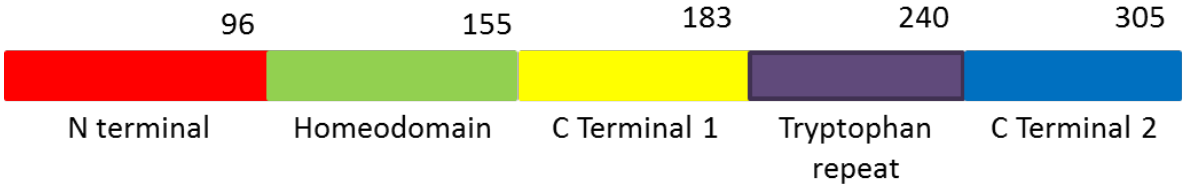


Figure 1-11. Schematic representation of Nanog protein. Nanog protein contains 3 main domains, N- terminal, the homeodomain and the C-terminal. The N-terminal is required for transcriptional activity, homeodomain for DNA interaction and the C-terminal contains transactivation domains.

A total of 12 Nanog genes have been identified to date within the human genome; one embryonic NANOG gene and eleven pseudogenes, with only 2 genes able to produce functional Nanog protein (Nanog and Nanog pseudogene 8) [156]. Nanog is located on chromosome 12 q3.13, whilst Nanog pseudogene 8 is located on chromosome 15 p13.31. Both proteins are identical with the exception of one amino acid (Glycine-253 in Nanog to Histidine-253 in Nanog P8) [156]. Notably, most commercially available anti-Nanog antibodies have been raised against Nanog and the similarity between the two proteins results in currently not being able to distinguish between them with certainty using anti-Nanog antibodies in western blotting or immunohistochemistry. In addition, though the predicted molecular mass of Nanog is approximately 35 kDa, when using commercially available anti-Nanog antibodies a varying molecular mass has been detected. One group, have detected Nanog at approximately 30-100 kDa, and Nanog P8 at 28-180 kDa, suggesting this is due to multiple conformations of Nanog and Nanog P8. A limited number of studies have used gene expression analysis to identify the presence of Nanog and Nanog P8 via PCR in immortalised cancer cell lines and solid human primary cancerous tissues. There have been suggestions that Nanog is expressed in high levels in human embryonic stem cells whilst Nanog P8 is expressed at high levels in human cancer cells. However, others have reported both Nanog and Nanog P8 in cancerous cells [153] [157]. The functional importance of Nanog and Nanog P8 has not been fully elucidated.

1.13.2 Role of Nanog in gastrointestinal cancers

1.13.2.1 Nanog and Nanog P8 in CRC

Nanog expression is variable across a range of CRC cell lines. In a panel of CRC cell lines, Nanog and Nanog P8 expression was detected by RT-PCR analysis (SW480, DLD-1, HCT116, HT29, RKO, SW620 and SW48). Nanog protein was either very low or undetectable in 2 cell lines (SW480 and DLD-1) [158]. In addition, Nanog was detected predominantly in SW620 cells, whilst Nanog P8 was most readily detectable in HT29 and HCT116 cells suggesting variability amongst cell lines. Another study using 6 different CRC lines, Nanog P8 was found to be the predominantly expressed form (Clone A, CX-1, KM-12c, MIP-12c, LS-174T, HCC-2998) in monolayer and spheroid culture suggesting Nanog P8 also contributes to stem-like properties [157]. Furthermore, in cancer cells Nanog was found to be localised to the cytoplasm, whereas in embryonic cells it was predominantly detected in the nucleus [158]. Interestingly, in a sample of 10 human CRC liver metastases, Nanog was detected in 8, of which 6 expressed Nanog

P8 [157]. These results indicate that Nanog and Nanog P8 are often expressed together, with a predominance of one in a range of CRC cell lines, however it is not clear how expression is regulated or the resulting impact on Nanog function.

1.13.2.2 Nanog and Nanog P8 Gastric cancer

Using a gastric cancer cell line (SGC-7901) over expression of Nanog P8 was associated with increased proliferation of cells, decreased apoptosis and increased cell invasion and migration [159]. In human gastric cancer patient tumours, Nanog and Nanog P8 are both expressed significantly higher compared to normal tissues. However, in an alternative study of 60 GI cancers, Nanog P8 was found to be expressed only in three patient samples which were felt to be in particularly infiltrative tumours. In this study though Nanog and Nanog P8 levels did not relate to the prognosis of patients with gastric cancer. Others have reported a correlation [160, 161]. In addition, authors did suggest that Nanog and Nanog P8 play a role in the precancerous phases of gastric cancer (intestinal metaplasia and dysplasia) and so could be a potential biomarker for predicting malignancy [161].

1.13.3 CRC CSC and Nanog signalling in-vivo models

Despite functional studies providing evidence Nanog may increase proliferation, increase tumour formation, links with metastasis and a possible association with resistance, the effect if Nanog in transgenic murine models has not been classical [150]. In a doxycycline-induced Nanog overexpression murine model higher levels of Nanog were detected in most tissues compared to control mice e.g. higher levels of Nanog expression were detected in cerebral, liver, kidney, heart, stomach, lung, liver, intestine, colon and spleen tissues [162]. However, following induction of Nanog over-expression, morphological changes of hyperplasia were detected but these were restricted to the small intestine and colon. Notably, these hyperplastic lesions did not develop into adenomas or colorectal malignancies. In addition, these changes reversed when the induction of Nanog overexpression was removed [162]. The hyperplastic lesions had higher levels of proliferation compared to control tissues and there was decreased gene expression of Cdx2 and Klf4. No change in the wnt pathway was detected as a result authors suggested that Nanog may influence tumours which are already present, rather that propagating tumours [162]. In a similar induction model, Nanog over expression led to hyperplasia in the oesophagus [163]. In contrast, when Nanog was over expressed in a murine model with wnt, mammary tumorigenesis and metastasises was observed

[147]. An alternative model, metastasis associated in colon cancer 1 (MACC1) was upregulated in a transgenic model with mice which harboured an *APC* mutation. Here tumours formed more readily than in control mice via the gene expression of Oct4 and Nanog. Moreover authors suggested MACC1's tumour effect maybe mediated via the MACC1/Nanog/Oct4 axis [164].

1.14 CSC targeting therapies

A number of early phase clinical trials are underway using novel agents, repurposed drugs and immunotherapies thought to target CSCs in a variety of solid tumours (see table 1-6). These studies highlight the current and increasing interest from the pharmaceutical industry and academia in developing therapeutic strategies to eradicate this sub-population of cells [165]. Many of these trials are still actively recruiting or are yet to publish results. Additionally, the studies involving novel agents are phase 1 trials investigating endpoints related to safety profile and pharmacokinetics; they do not include measuring specific effects on the CSC population as a trial endpoint.

One study, which aimed to investigate the use of metformin against colorectal cancer CSCs in a window trial, and has reported preliminary results suggesting biological activity (NCT01440127); metformin (500mg) taken twice a day for two weeks prior to scheduled surgery decreased the expression of the stem cell markers CD133, Oct4 and Nanog in colorectal cancer tissue compared to levels in tissue from control participants [166]. Another notable study is specifically looking at the use of a check point blockade immunotherapy (iplimumab) and EGFR blockage (cetuximab) and its effect on the CSC regulating pathway, hedgehog. This is a Phase 1 study, primarily considering safety, however, serial tumour biopsies were carried out allowing clinical responses to be correlated with pathways of interest. The authors demonstrated that a clinical response may correlate with the down regulation of EGFR and hedgehog pathways. Of the nine patients who were treated, one had a partial response, four had stable disease and three had progressive disease. One patient was not assessed due to toxicities associated with cetuximab.

Interestingly, chemotherapies are also being evaluated for their CSC targeting properties e.g. mithramycin.

Table 1-6. Clinical trials using agents targeting cancer stem cells.

A variety of therapies are currently being investigated in early phase clinical trials. These include novel agents such as R04929097 which targets the notch signalling pathway, repurposed drugs such as metformin and bevacizumab and immunotherapies such as dendritic cell vaccines.

Agent	Cancer Type	Trial Name	Trial Number
MK0752	Breast	Phase 1/2 study of MK0752 and docetaxel in advanced breast cancer	*NCT00645333*
	Pancreatic	Phase 1 study of MK0752 and gemcitabine in pancreatic cancer	*NCT01098344*
	Pancreatic	MK0752 and gemcitabine in patients with unresectable pancreatic cancer	*NCT01098344*
	Breast	Pilot study of MK0752 and tamoxifen or letrozole in patients with breast cancer prior to surgery	*NCT00756717*
	Solid Tumour	Phase 1 study of MK-8669, MK-2206 and MK-8669 or MK0752 in patients with advanced cancer	*NCT01295632*
	CNS tumours	MK0752 in young patients with CNS cancer	*NCT00572182*
	Breast/Solid Tumour	Phase 1 study of MK0752 in patients with metastatic or locally advanced breast cancer or solid tumours	*NCT00106145*
RO4929097 **Only colorectal cancer studies shown***	Colorectal	Phase 1 study of cetuximab and R04929097 in metastatic colorectal cancer	*NCT01198535*

	Colorectal	Phase 2 study of RO4929097 (NSC 749225) and FOLFOX with bevacizumab versus FOLFOX and bevacizumab for the first-line treatment of patients with metastatic colorectal cancer	*NCT01270438*
	Colorectal	Phase 2 study of RO4929097 in metastatic colorectal cancer	*NCT01116687*
Metformin	Ovarian/ Peritoneal	Phase 2 study investigating metformin in the prevention of relapse in patients with advanced ovarian, fallopian tube and primary peritoneal cancer	*NCT01579812*
	Colorectal	Window study assessing effect of metformin pre-treatment on colorectal cancer stem cells and related pharmacodynamic markers in patients undergoing colorectal cancer resection.	*NCT01440127*
	Solid Tumour	Phase 1 study assessing safety of metformin with chemotherapy in patients with solid tumours	*NCT01442870*
	Pancreatic	Window study assessing effect of metformin pre-treatment on pancreatic cancer stem cells and related pharmacodynamic markers in patients undergoing pancreatic resection.	*NCT01954732*
	Liver	Phase 1 study assessing safety of metformin with chemotherapy in patients with liver cancer	*NCT01442870*
Reparixin	Breast	Phase 1 study of reparixin and paclitaxel in patients with HER2 negative metastatic breast cancer	*NCT02001974*
	Breast	Window study assessing effect of reparixin pre-treatment on colorectal cancer stem cells and related pharmacodynamic markers in patients undergoing breast cancer resection	*NCT01861054*

	Breast	Phase 1 study of paclitaxel with reparixin or placebo for metastatic breast cancer	*NCT02370238*
Carbohydrates	Colorectal	Effect of non-digestible carbohydrates on biomarkers related to colorectal cancer	*NCT01214681*
OMP 21M18	Colorectal	Phase 1 study of FOLFIRI and OMP 21M18 in patients with metastatic colorectal cancer	*NCT01189942*
	Solid Tumours	Phase 1 study of OMP 21M18 in patients with solid tumours	*NCT00744562*
	Pancreatic	Phase 1 study of gemcitabine OMP 21M18 with or without abraxane in patients with advanced pancreatic cancer	*NCT01189929*
	Lung	Phase 1 study of carboplatin and pemetrexed and OMP 21M18 in patients with non-small cell lung cancer	*NCT01189968*
	Ovarian/ Peritoneal	Phase 1/2 study of OMP 21M18 and paclitaxel in patients with platinum resistant advanced ovarian, fallopian tube and primary peritoneal cancer	*NCT01952249*
Bevacizumab	Breast	Phase 2 study evaluating cancer stem cell activity of pre-operative bevacizumab and chemotherapy in patients with breast cancer	*NCT01190345*
Dendritic Cell Vaccine	Glioblastoma	Phase 1 study of vaccination with autologous dendritic cells pulsed with lysate derived from allogeneic glioblastoma stem- like cell line for patients with a new diagnosis of or recurrent glioblastoma.	*NCT02010606*
	Glioblastoma	Phase 1/2 study of vaccine therapy with stem cell derived mRNA-transfected dendritic cells in patients with glioblastoma	*NCT00846456*
Ipilimumab	Head and neck	Phase 1 study of cetuximab and IPI-926 (ipilumumab) in recurrent head and neck cancer	*NCT01255800*
	Upper thorax malignancies	Phase 2 study of mithramycin, an Inhibitor of cancer stem cell	*NCT01624090*

Mithramycin		signalling, in patients with malignancies involving lungs, oesophagus, pleura, or mediastinum	
Vismodegib	Pancreatic cancer	Pilot study of vismodegib (hedgehog inhibitor) in combination with gemcitabine	NCT01195415

Abbreviations: Folinic acid, Oxaliplatin, Fluorouracil (FOLFOX), Folinic acid, Irinotecan, Fluorouracil (FOLFIRI)

1.15 Aims and Objectives

Unpublished data has shown curcumin is able to decrease the proliferation of CSCs derived from human CRC and adenoma tissue (figure 1-12). In addition, in NOD-SCID mice bearing xenografts from human CRC CSCs, defined by $ALDH^{high}$ curcumin significantly delayed the growth of tumours and improved survival (figure 1-13). Curcumin also decreases the number of CSCs in intestines of *ApcMin* mice compared to control animals, which correlates with therapeutic prevention efficacy in this model. Data reproduced with kind permission from Dr A Karmokar [167].

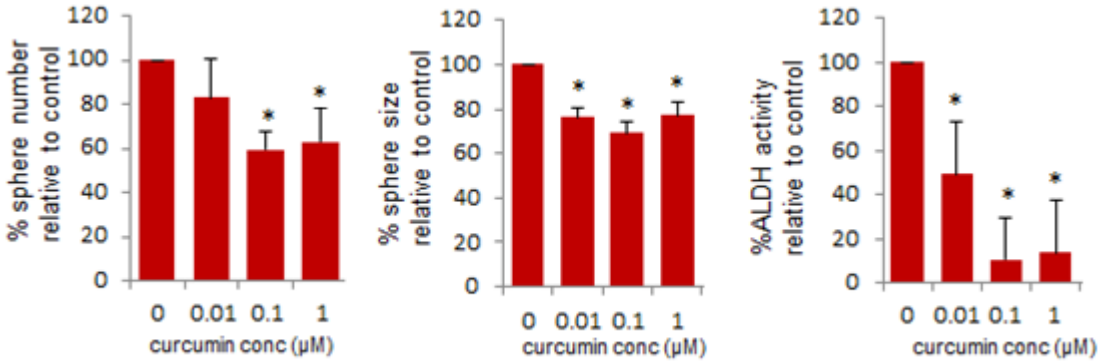


Figure 1-12. Curcumin caused a significant reduction spheres formed from CRC and adenoma samples. The optimal concentration varied between patients. Results are representative of response observed with 3 adenoma and 3 cancer samples. Data reproduced with kind permission from Dr A Karmokar [167].

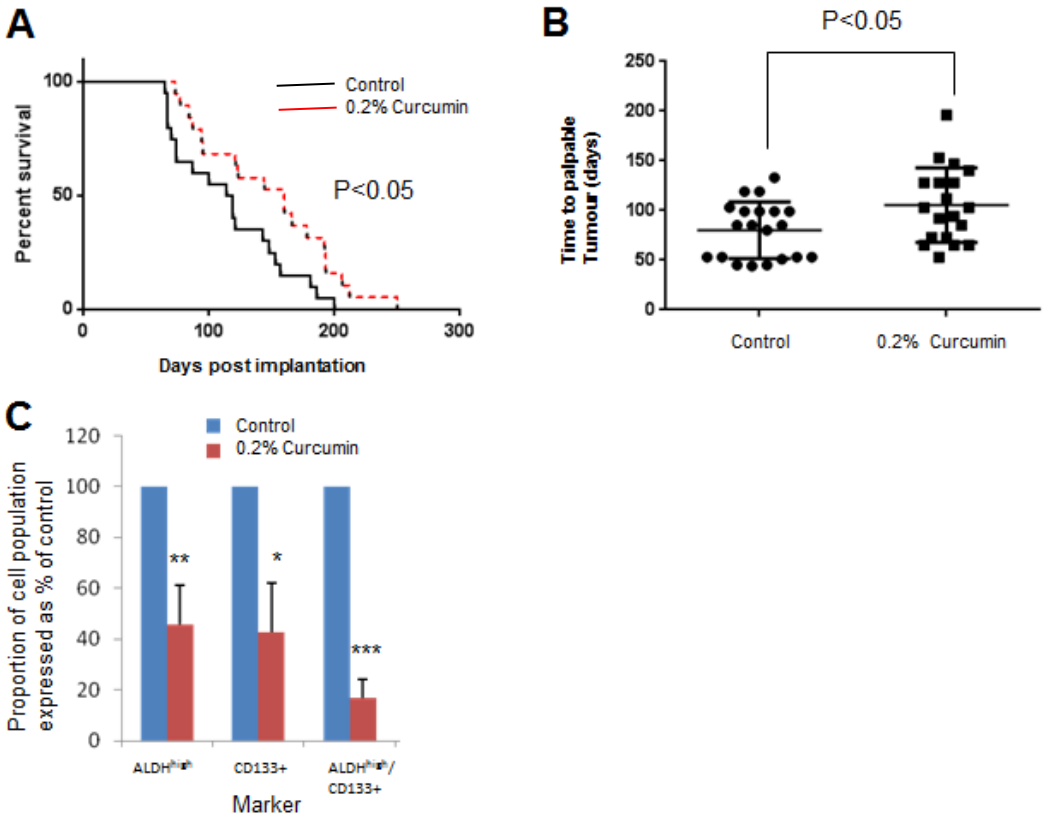


Figure 1-13. Effect of curcumin on tumour development in NOD/SCID mice following implantation of ALDH^{high} cells isolated from a human CRC sample. (A) Survival of NOD/SCID mice consuming 0.2% curcumin in the diet was significantly increased compared to control animals (p=0.03). (B) Time taken to palptate tumours was significantly slower in mice given curcumin compared to control mice. (C) The proportion of tumour cells taken from mice staining for high ALDH activity or CD133 expression or for both (ALDH^{high}/CD133^{+}) was significantly reduced in the curcumin arm. (A total of 19 mice were given 0.2% curcumin diet and 20 mice given control diet/ *p<0.05, p**<0.01, ***p<0.001 as determined by Students T-test). Data reproduced with kind permission from Dr A Karmokar [167].

The mechanism of action remains unclear, though links with Nanog have been made. The aims of this project were to establish if Nanog is a suitable target for CRC prevention strategies. The hypothesis is curcumin can target Nanog in human colorectal cancer tissues. Specifically, the objectives were:

1) Characterisation of Nanog expression in human tissues (Chapter 3 results and discussion)
2) Assess the effect of curcumin on Nanog in colorectal cancer cell lines (Chapter 4 and 5 results and discussion)
3) Determine whether Nanog is identifiable in human CRC explant tissues and targetable by curcumin treatment (Chapter 6 results and discussion)

Overall, it is hoped the results from this thesis will help to identify potential markers of curcumin efficacy in CRC. This will lead to the development of biomarkers which can help design and direct future clinical trials.

2.0 Chapter 2 - Materials and Methods

2.1 Maintenance and passaging of immortalised cell lines

2.1.1 Caco-2 colorectal cancer cell line

Caco-2 cells were purchased from ATCC (UK). Caco-2 cells represent a moderately well differentiated colorectal adenocarcinoma adherent immortalised cell line, originating from a 72 year old man. Cells were maintained in minimal essential medium (MEM) (Sigma, UK), supplemented with 20% foetal calf serum (FCS) and 1x glutamax (Gibco, UK) in an incubator at 37°C with 5% CO_2 in a humidified atmosphere. Cells were passaged when reaching 70-80% confluency and did not exceed passage 25. When passaging, media was aspirated from the flask, cells were washed twice with phosphate buffered saline (PBS) prior to the addition of 3x trypsin/ethylenediaminetetraacetic acid (EDTA) for 5 minutes at 37°C. Subsequently, an equal volume of serum-containing medium was added to neutralise the trypsin. Cells were then centrifuged at 1300 rpm (~400 *x* g) for 3 minutes and washed with PBS prior to resuspension in medium. They were counted using a Z2 particle counter (Beckman Coulter) and replated at the density required. Cells were regularly tested for mycoplasma contamination.

2.1.2 Hela cervical cancer cell line

HeLa cells were maintained and passaged in the same manner, using dulbecco's modified eagle medium (DMEM) (Sigma, UK) supplemented with 10% FCS. Cells were purchased from ATCC (UK) and donated to the project by Dr Don Jones (Professor in Translational Biomarkers, Leicester Cancer Research Centre, University of Leicester). HeLa cells were used as a positive control in sequencing experiments and represent a cervical adenocarcinoma adherent cell line originating from a 31 year old woman. Cells were regularly tested for mycoplasma contamination and did not exceed passage 25.

2.1.3 HCT116 colorectal cancer cell line

$HCT116^{GFP}$ and $HCT116^{GFP/Nanog}$ colorectal cancer cell lines were donated to this project by Dr Abdolrahman Shams-Nateri (Associate professor in Cancer Genetics and Stem Cells, Faculty of Medicine and Health Sciences, University of Nottingham). CRC cell line HCT116 (s45 beta-catenin mutant) overexpressing the green fluorescent

protein/plasmid control ($HCT116^{GFP}$) or cells with overexpression of human Nanog (NANOG1-promoter-GFP-postive fusion protein) ($HCT116^{GFP/Nanog}$) were used [168] [169]. Cells were maintained using RPMI-1640 medium (Sigma, UK) supplemented with 10% FCS and 1x glutamax (Gibco, UK) in an incubator at 37°C with 5% CO_2 in a humidified atmosphere. Cells were selected using 5 μg/ml Puromycin (Invitrogen, UK) for 2 weeks to maintain the highest levels of Nanog. Selection was re-applied every 2-4 weeks. Cells were originally taken from a 48 year old male with ascending colon adenocarcinoma. They were passaged in a similar manner to caco-2 cells and were regularly tested for mycoplasma contamination. Cells did not exceed passage 25. Mutational profiles of colorectal cancer cell lines used are described in table 2-1.

Table 2-1. Colorectal cancer cell lines classified by molecular pathways CIN, MSI and CIMP and mutation status of important cancer related genes.

(Modified from [170])

Cell line	CIMP panel 1	CIMP panel 2	CIN	KRAS	BRAF	PIK3CA	PTEN	TP53	MSI status
Caco2	+	-	+	WT	WT	WT	WT	E204X	MSS
HCT116	+	+	-	G13D	WT	H1047R	WT	WT	MSI

Abbreviations: CpG island methylator phenotype (CIMP), Chromosomal instability pathway (CIN), Kirsten rat sarcoma virus (KRAS), Proto-oncogene serine/threonine kinase (BRAF), Phosphatase and tensin homolog (PTEN) and tumour protein 53 (TP53).

2.2 Obtaining primary colorectal cancer and adenoma tissues and paired blood samples

CRC tissues were obtained as part an ethically approved study 'Development and application of *ex vivo* assays to assess efficacy biomarkers in the prevention and treatment of cancer' (UNOLE 0472). Ethical approval was granted by Wales Research Ethics Committee 4 (REC reference 14/WA/1166). Collection and use of tissues is summarised in figure 2-1. All tissues were collected by trained laboratory staff and remained anonymised to the researchers. Briefly, samples were collected from patients undergoing surgical resection for colorectal cancer. Tissue samples were collected

directly from theatre and transported on ice to the pathology department at Leicester Royal Infirmary. The sample was processed by a pathologist or biomedical scientist and any excess tissue offered for experimental purposes. Excess tissue was placed directly into 10 mL of media 199 containing antibiotic/antimycotic (Gibco, UK) on ice. Tissue was used to create a single cell suspension for baseline expression of CSC markers and sphere forming assays, kept intact for explant tissue culture and formalin fixation depending on quantity/quality of tissue and experiment planned. Blood samples were taken pre-procedure. Samples were obtained with Leicester Experimental Cancer Medicines Centre (ECMC) and University Hospitals of Leicester (UHL) support. Example Haematoxylin- eosin (H and Es) of tissues are shown (figure 2-2).

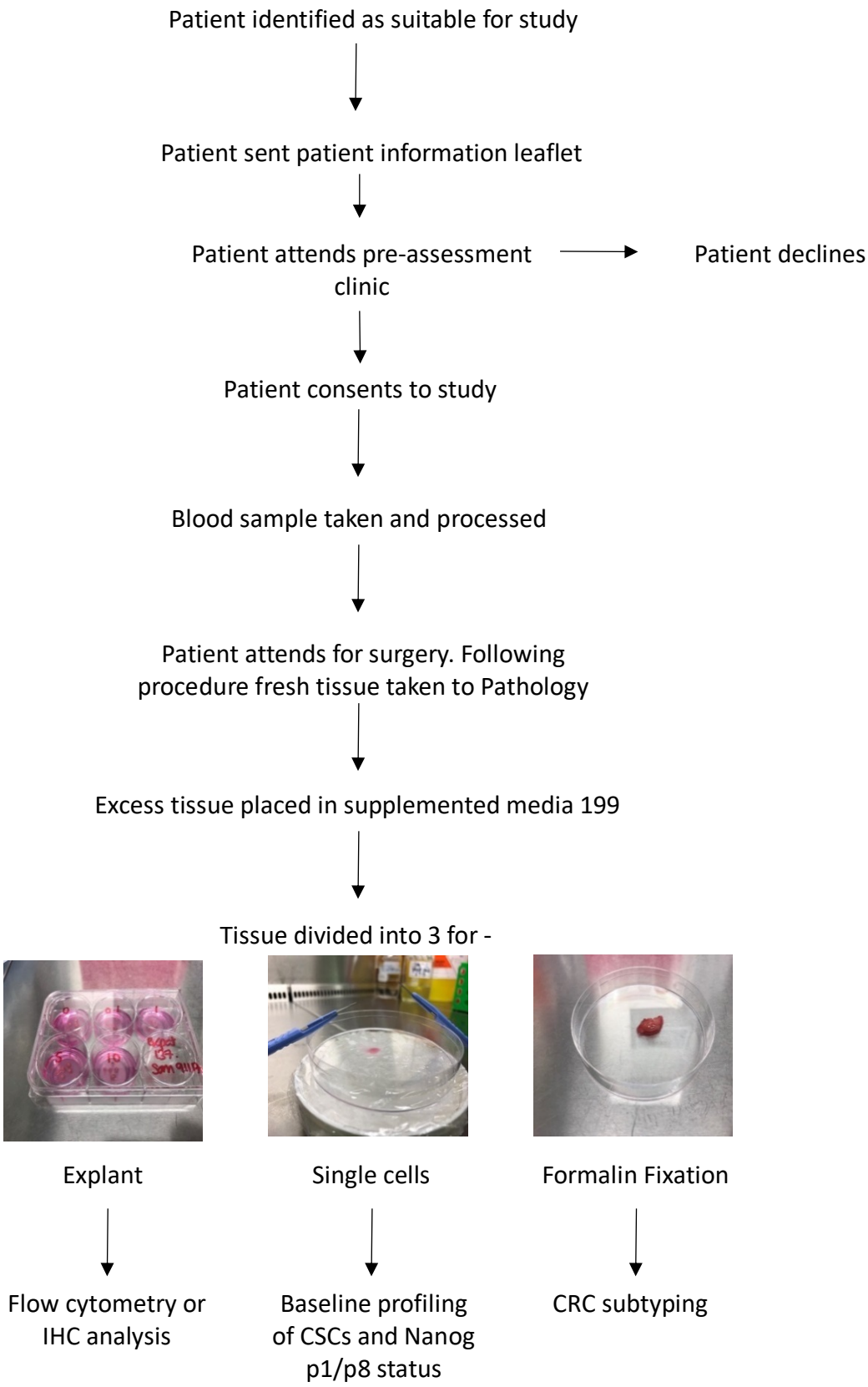
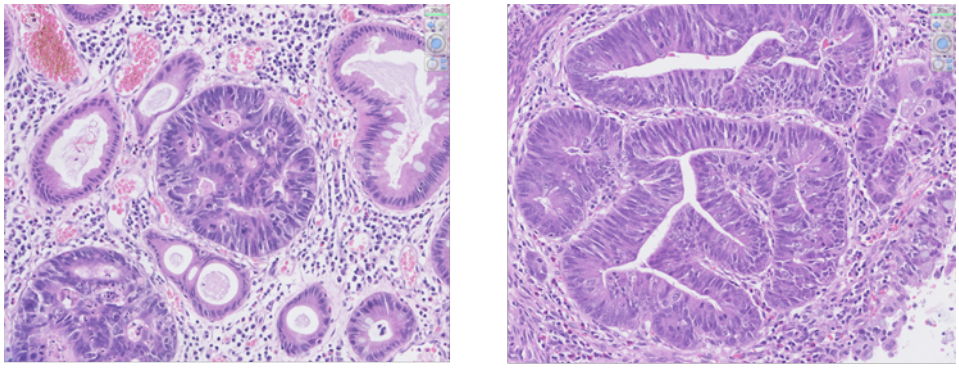


Figure 2-1. Tissue pathway outline. Patients were identified as suitable for study and sent a patient information leaflet. Following surgery excess tissue was used for explants, single cells or formalin fixation.



Adenoma tissue Cancer tissue

Figure 2-2. Example H and E's of tissues obtained for use (x20). Tissues were collected as described and H and E's carried out to ensure tissues were histologically confirmed as normal, adenoma or cancerous tissues.

2.3 Creating a single cell suspension from human tissues – baseline expression of CSC markers

Tissue placed in media 199, were bleached for 3 seconds in a solution to eliminate any bacterial or fungal contamination (308 μL of 13% sodium hypochlorite in 250 mL water). The tissues were then put into 5 mL of media 199 and minced into pieces with two sterile scalpels. A further 5 mL of media 199 and collagenase type 4 (Worthington Chemicals, UK) was added. The suspension was incubated at 37°C for 60 minutes, with cells being pipetted every 15 minutes. The cells were then passed through a 100 μm filter (BD Falcon, UK), cells centrifuged and washed with HBSS (Sigma, UK) and passed through a 40 μm filter. Cells were washed a further two times with HBSS and counted using a haemocytometer. Cells ($1x10^6$/vial) were frozen in freezing media (90% FCS and 10% dimethyl sulfoxide (DMSO)) and stored long-term in liquid nitrogen for flow cytometric analysis.

2.3.1 Fluorescence-activated cell sorting (FACS) analysis and sorting conditions

FACS analysis and sorting was carried out using a BD FACS Aria II (85 μM nozzle). Cytometer set up and tracking was carried out prior to all sorts and analyses. Cell sorts were carried out under sterile conditions, and sorted cells were collected into tubes containing sterile staining buffer. Cells were gated and collected. For experiments

requiring more than one antibody, appropriate compensation was set up and applied to minimise spectral overlap between fluorescence signals from respective fluorochromes.

2.3.2 Staining of single cells for Nanog$^+$, ALDH1A1$^+$, Ki67$^+$ and Caspase$^+$ using a flow cytometry based assay

Single cells were harvested and suspended in PBS (up to 1x10^6/mL). Cells were fixed and permeabilised using cell signalling buffer set A (Miltenyi Biotech, UK). Following two washes with PBS supplemented with 0.5% BSA and 2 mM EDTA at pH 7.2 (buffer), fluorescein isothiocyanate (FITC) conjugated mouse anti- human ALDH1A1 antibody was added to a final concentration 1:100 (Abcam, UK), allophycocyanin (APC)-conjugated mouse anti-human Nanog antibody was added to a final concentration 1:11 (Miltenyi Biotech, UK), phycoerythrin (PE)-conjugated mouse anti-human ki67 antibody was added to a final concentration of 1:11 (Miltenyi Biotech, UK) and bright ultra violet (BUV)395-conjugated rabbit anti-human caspase-3 antibody (BD, UK). Anti-human IgG1 negative control was used to define THE Nanog population of interest (Miltenyi Biotech, UK). Cells were incubated for 30 minutes at room temperature in the dark. Subsequently, 2 washes were performed, and cells were suspended in 200 µL of buffer prior to analysis using a using a BD FACS Aria II. FACSDiva version 6.1.3 was used for data acquisition and analysis, with a minimum of 10000 events for each sample.

The gating strategy used to identify the CSC and non-CSC population is described in figure 2-3. Briefly using the side scatter and forward scatter in a two parameter density plot viable single cells were identified. This helped to eliminate debris, dead cells and clumps from analysis. Doublets were also specifically removed (2-3a). Single peaks were identified following anti-ALDH1A1 FITC, anti-ki67 PE and anti-caspase-3 BUV395 analysis (2-3b). Identification of anti-Nanog cells which represented our population of interest were defined using an isotype control (human IgG1 negative control). This helped to exclude any background staining or non-specific staining (2-3c). Fluorescence minus one (FMO) controls were used to help minimise spectral overlap and where enough material allowed.

Subsequently, using a bivariate fluorescence histogram the CSC population was identified (anti-ALDH1A1 in the FITC channel on the *y* axis and anti-Nanog on the *x* axis). This allowed Nanog$^+$, ALDH1A1$^+$, proportion of double positive CSC population cells (ALDH1A1$^+$Nanog$^+$) and non-CSC population cells (ALDH1A1$^-$Nanog$^-$) to be

identified (see figure 2-4a). The proliferating proportion of cells was also analysed via a second bivariate fluorescence histogram (anti-Nanog in the APC channel on the *y* axis and anti-ki67 in the PE channel on the *x* axis) (see figure 2-4b). The apoptotic proportion of cells was also analysed via a third bivariate fluorescence histogram (anti-Nanog in the APC channel on the *x* axis and anti-caspase-3 in the BUV395 channel on the *y* axis) (see figure 2-4c).

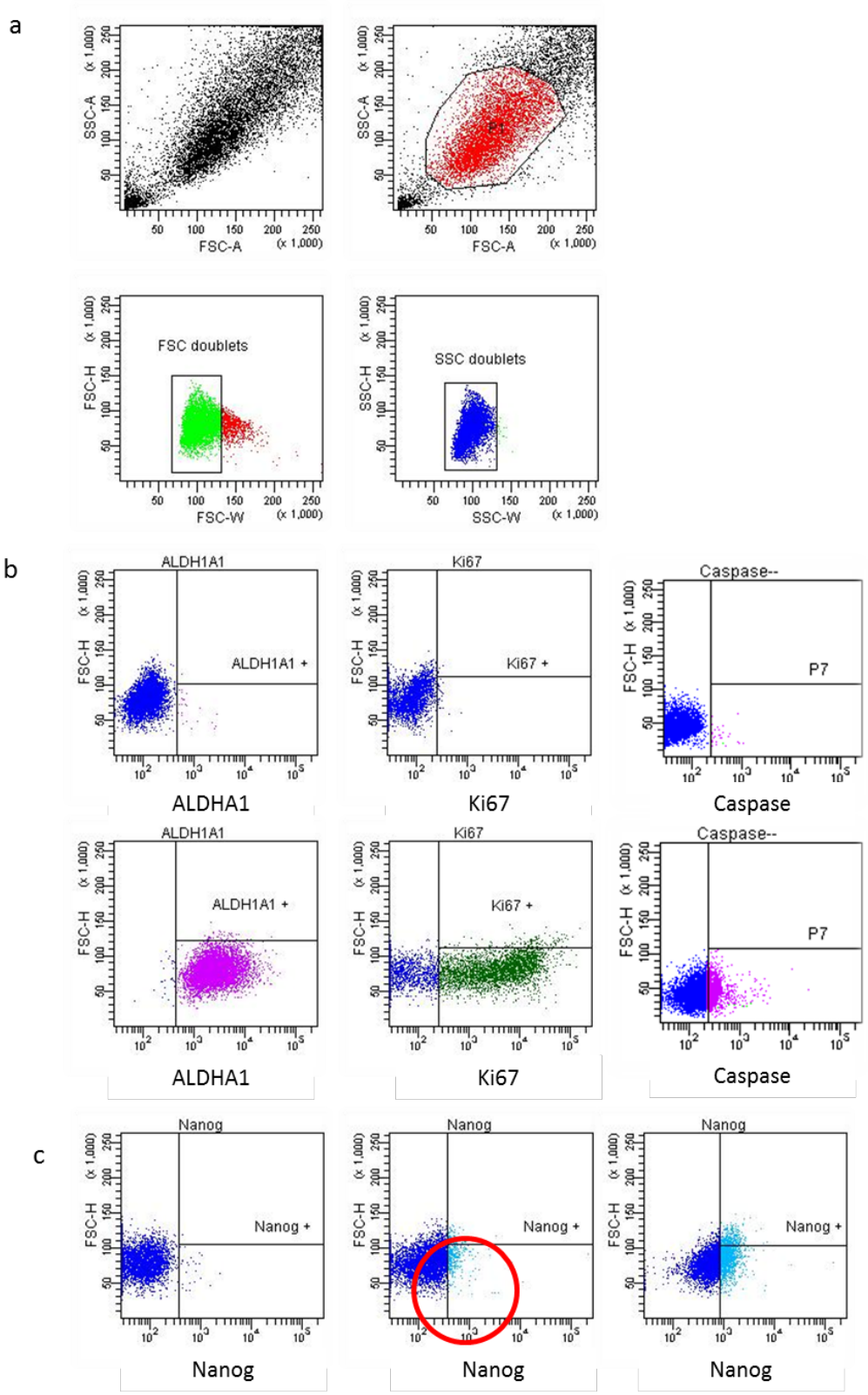


Figure 2-3. Gating strategy used to identify CSC, non-CSC population, CSC proliferation and CSC apoptosis. (a) Identification of viable cells. (b) Identification of ALDH1A1^{+}, Ki67^{+} and Caspase^{+} cells. (c) Identification of Nanog^{+} cells (isotype circled in central panel resulting in shift of gate to the right).

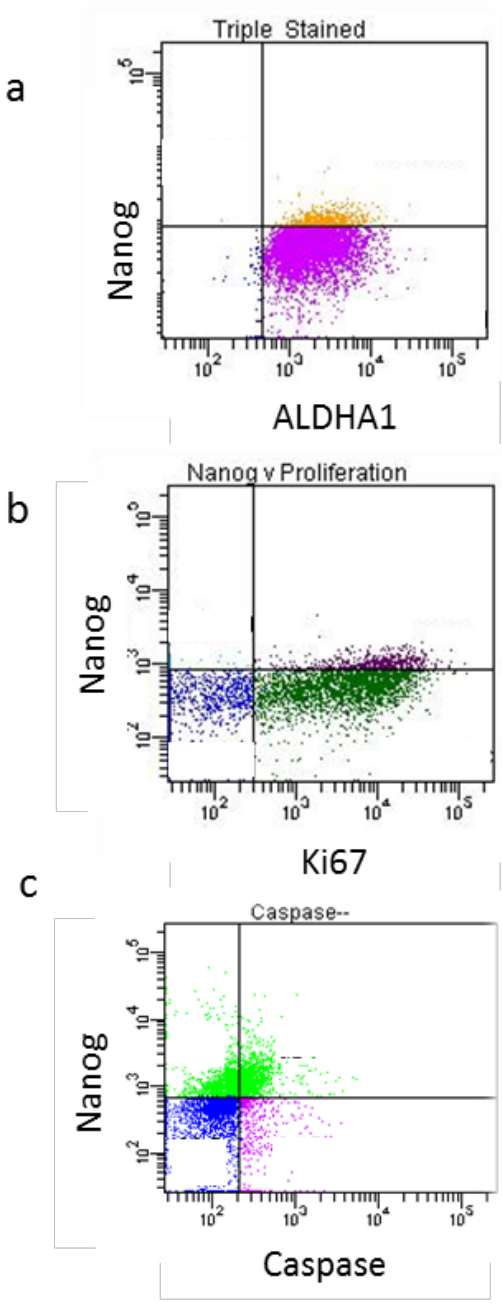


Figure 2-4. Gating strategy used to identify CSC, non-CSC population, CSC proliferation and CSC apoptosis. (a) Identification of $Nanog^{+}ALDH1A1^{-}$, $ALDH1A1^{+}Nanog^{+}$, $Nanog^{+}ALDH1A1^{+}$ and $Nanog^{-}ALDH1A1^{-}$ cells (b) Identification of $Nanog^{+}Ki67^{+}$, $Nanog^{-}Ki67^{+}$ and $Nanog^{-}Ki67^{-}$ cells. (c) Identification of $Nanog^{+}Caspase^{+}$ and $Nanog^{-}Caspase^{+}$.

2.3.3 Staining of single cells for ALDH activity, CD133$^+$ and ESA$^+$ using flow cytometry based assay

The aldefluor assay kit (Stem Cell Technologies, UK) was used as per manufacturers' instructions (figure 2-5). To prepare the aldefluor substrate, the inactivate substrate was dissolved in 25 μL DMSO and mixed for 1 minute. Subsequently 25 μL 2N hydrochloric acid was added to the solution and incubated for 15 minutes at room temperature. Lastly, 360 μL of buffer was added. The resulting activated mixture was aliquoted and stored at -20°C. For staining, cells were suspended in 1 mL buffer. Cells were split into 2, with 500 μL added to 10 μL ALDH inhibitor, diethylaminobenzaldehyde (DEAB). This was used as a negative control to aid gating. Immediately thereafter 2.5 μL of activated substrate was added. The remaining 500 μL of cells were added to 2.5 μL of activated substrate and mixed well. This sample was used to test for ALDH activity. Both samples were incubated at 37°C for 40 minutes. The activated substrate was converted to a negatively charged substrate by intracellular ALDH within cells. The negative charge prevents diffusion out of the cell, however it can be effluxed from cells by the ATP binding cassette transporter system. This is prevented by the buffer provided in the kit.

Formerly, different approaches have been used to quantify ALDH in cells and tissues, with early methods relying on enzyme assays or western blotting. However, these methods did not accurately measure different isoenzymes due to antibody cross-reactivity. The specific isoform used by BD biosciences clone 44 or Abcam ab52492 is directed against ALDH1 is ALDH1A1 [145]. Currently the aldefluor assay is used to measure ALDH activity in viable cells using flow cytometry. Expression of ALDH does not necessarily equate to ALDH activity, therefore the aldefluor assay allows more accurate identification of CSCs [145]. This highlights a discrepancy between experimental approaches and ambiguities in reported data.

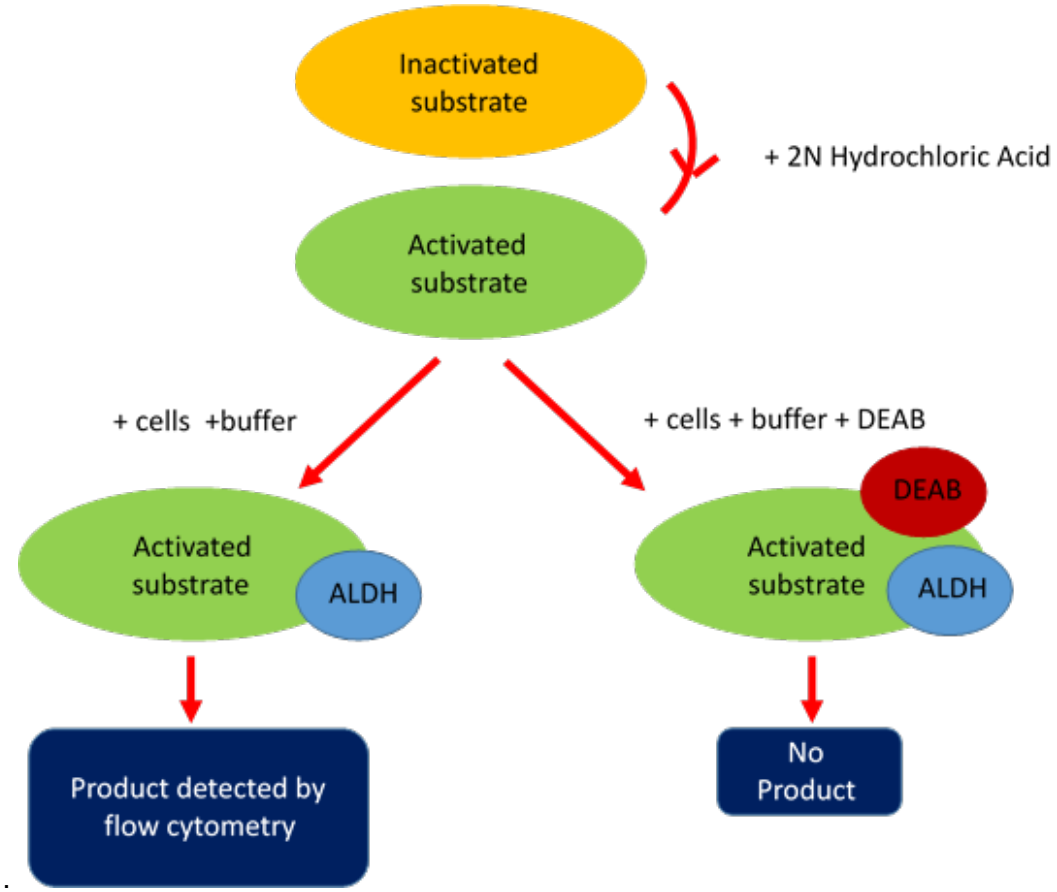


Figure 2-5. Representation of Aldefluor assay. Inactivated substrate (orange) is activated following addition of 2N hydrochloric acid. This turns the substrate a fluorescent green, allowing easy detection of activated substrate. Buffer is added which contains an inhibitor of the ATP-binding cassette (ABC) system, therefore maintaining product within cells. The product can then be detected by a flow cytometer. DEAB inhibits ALDH, therefore no product is detected. This population of cells is used as a negative control to aid gating.

Following ALDH staining, cells were washed in buffer and resuspended in 80 μL. Subsequently, 10 μL APC-conjugated mouse anti-human CD133 and PE-conjugated mouse anti-human ESA antibody was added (Miltenyi Biotec, UK). The cells were incubated in the dark for 30 minutes at 4°C, washed and resuspended in 300 μL of buffer prior to analysis using a using a Becton Dickinson (BD) Aria II. FACSDiva version 6.1.3 was used for data acquisition and analysis, with 10000 events for each sample.

The gating strategy used to identify the CSC population based on ALDH activity is described in figure 2-6. Viable cells were identified as described previously. For each sample to be tested for ALDH activity, an ALDH inhibitor, diethylaminobenzaldehyde (DEAB) was used as a negative control. As ALDH activity is a spectrum of activity from low to high, strict criteria were applied to ensure only $ALDH^{high}$ (top 5%) and $ALDH^{low}$

cells (bottom 10%) were gated or sorted. Single peaks were identified following anti-ESA PE and anti-CD133 APC analysis. Initially ESA^+ cells were identified. Following this a bivariate fluorescence histogram was used to identify the $ALDH^{high}/CD133^-$, $ALDH^{high}/CD133^+$ and $ALDH^{low/}CD133^+$ populations (anti-ALDH in the FITC channel on the *x* axis and anti-CD133 on the *y* axis). This again is similar to section 3.3.2.

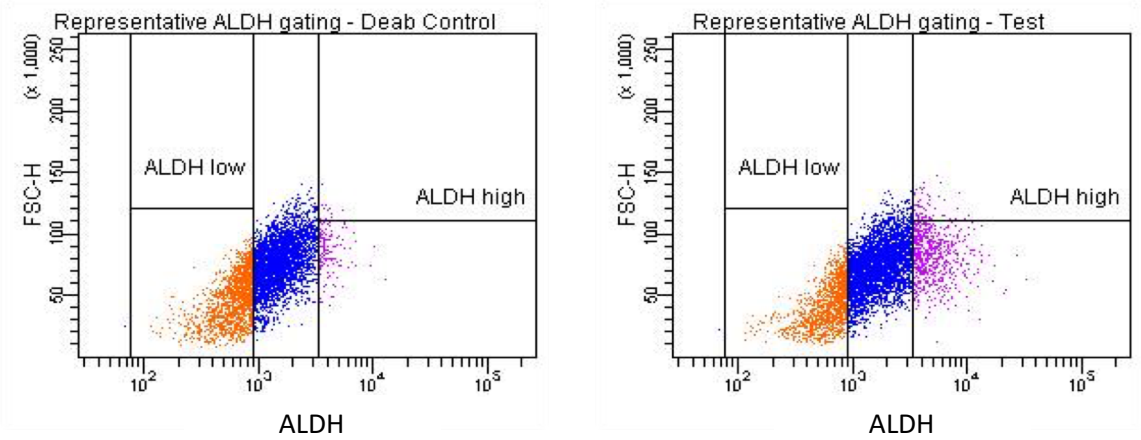


Figure 2-6. Gating strategy used to identify $ALDH^{high}$ and $ALDH^{low}$ CSC populations. DEAB control was used as a negative control to identify $ALDH^{high}$ cells (purple) (left). Stringent gating such as this yielded an $ALDH^{high}$ population of <5-10% (purple) (right).

2.4 Growth and maintenance of primary colorectal cancer stem cells

Following creation of a single cell suspension, cells were then seeded into ultra-low attachment plates for sphere growth in sphere medium (see table 2-2). Spheres were maintained by collecting spheres and then centrifuging at 1200 rpm (~400 *x* g) for 10 minutes at 4°C. The supernatant was aspirated and 3 mL 7.5x trypsin/EDTA added and mixed well prior to incubation for 10 minutes at 37°C. An equal volumes of media containing 20% FCS was added to neutralise the trypsin. Again, cells were mixed well. Single cells were washed in PBS and suspended in sphere medium prior to use.

Table 2-2. Overview of reagents required for 50 mL sphere medium.

Reagent	Volume
50% Neuroblast Medium (Fisher Scientific, UK)	25 mL
1% N-2 supplement (Fisher Scientific, UK)	0.5 mL
2% B-27 supplement (Fisher Scientific, UK)	1 mL
2% Antibiotic-Antimycotic (Fisher Scientific, UK)	1 mL
2 μg/mL Heparin (Sigma, UK)	50 μL
20 ng/mL FGF-2 (Fisher Scientific, UK)	10 μL
20 ng/mL EGF (Fisher Scientific, UK)	10 μL
DMEM/F12 medium 1:1 Hyclone (Fisher Scientific, UK)	22.5 mL

Abbreviations: Fibroblast growth factor- 2 (FGF-2), Epidermal growth factor (EGF), Dulbecco's modified eagle medium (DMEM).

2.4.1 Long-term sphere forming assays including measurement of sphere number and size

Primary single cells were plated at a density established following optimisation. Briefly, cells were plated between 10-60000 cells/well in a six well ultra-low attachment plate in sphere media and maintained for 4 weeks. The optimum density was picked for each primary sample and used for experimentation. Primary spheres were treated 3 times a week for 4 weeks prior to analysis. For treatments, curcumin was added to achieve a final concentration of 0-10 μM or DMSO was added. All wells were treated with equivalent concentrations of DMSO which did not exceed 0.1% DMSO.

All treatments were done in triplicate and repeated on 3 different occasions. For measurement of sphere number and size, a wax barrier was drawn around gridded slides. Spheres from each well were collected and loosely pelleted at 1200 rcf (~400 *x* g) for 10 minutes. The supernatant was aspirated with 30 μL left. This was used to resuspend the sphere pellets which were placed onto the waxed gridded slides. Cells

were counted and size measured using an inverted light microscope at 10x and 40x optical zoom respectively (Nikon Eclipse TE2000U) by a blinded assessor.

2.4.2 Staining of spheres for Nanog$^+$, ALDH1A1$^+$ and Ki67$^+$ or ALDH activity, CD133$^+$ and ESA$^+$ using flow cytometry based assay

Following treatments, cells were collected and single cells created using trypsin/EDTA as previously described. Subsequently, the treatment effects of curcumin on CSCs was assessed using flow cytometry.

2.5 Explant culture

Tissues placed in media 199, were bleached for 3 seconds in a solution to eliminate bacterial or fungal contamination. They were then cut into 1-2mm^3 pieces ensuring maintenance of tissue integrity and allowing compounds of interest to infiltrate the tissue. Tissues were then placed on to inserts (45 μM) in wells containing 1500 μL explant media (DMEM (Sigma, UK) containing 1% antibiotic/antimycotic and 2% FCS. Following a period of resting overnight, explants were treated for 24 hours with a range of curcumin doses (0-10 μM) prior to harvesting. Explants were analysed for protein expression, gene expression or CSC marker expression via immunohistochemistry (IHC) (figure 2-6) or flow cytometry (figure 2-7) respectively.

2.5.1 IHC using explant tissues - Nanog, ALDH1A1, Ki67, Caspase-3 and Mucin 2

Paraffin-embedded sections were dewaxed by heating to 65°C for 20 minutes and then hydrated in a series of 3 minute washes starting with 100% xylene then 99% industrial methylated spirit (IMS) and finally 95% IMS. Antigen retrieval was performed by microwaving in citrate buffer pH 6.0 (Sigma, UK) for 20 minutes at 99°C and allowed to cool to room temperature. Endogenous activity was blocked using Bloxall blocking solution (Vector Labs, UK) for 10 minutes at room temperature. Subsequently, three washes were performed in PBS and tissues were blocked using 2.5% normal horse serum (NHS) (Vector Labs, UK) for 20 minutes at room temperature. Nanog antibody (Novus biologicals, UK) was diluted 1:5000 in 2.5% NHS, ALDH1A1 antibody (BD Pharminogen, UK) was diluted 1:100 in 2.5% NHS or Mucin 2 antibody (Abcam, UK) was diluted in 1:2000 in 2.5% NHS and incubated overnight at 4°C. Slides were washed in PBS three times and Immpress reagent (Vector Labs, UK) added for 30 minutes at

room temperature. Slides were washed again in PBS three times and DAB substrate (Vector Labs, UK) added for 2 minutes (Nanog antibody or MUC2 antibody) or VIP substrate (Vector Labs, UK) added for 4 minutes (ALDH1A1 antibody). Slides were washed again in PBS, rinsed in water and counter stained with haematoxylin (Leica Biosystems, UK) for 30 seconds. Slides were rinsed in water and dehydrated in graded IMS and cleared in xylene prior to being mounted permanently with DPX mounting solution.

Caspase-3 and Ki67 analysis was carried out by Dr Leah Officer (Histology Research Officer, MRC Toxicology Unit).

2.5.2 Flow cytometry with explant tissues – Nanog$^+$, ALDH1A1$^+$ and Ki67$^+$

Single cells were created from 1-2mm^3 pieces and stained as previously described.

2.6 RNA extraction and elimination of genomic DNA

Total RNA was isolated from cell pellets using an RNeasy mini kit (Qiagen, UK) according to manufacturers' instructions. Pellets were lysed using RLT buffer before homogenising using the QIAshredder spin kit (Qiagen, UK). Genomic DNA contamination was eliminated using the RNase free DNase kit (Qiagen, UK) following manufacturers' instructions. Total RNA was eluted in a final volume of 30 μL nuclease free water (Invitrogen, UK). RNA was quantified using a NanoDrop spectrophotometer (Nanodrop Technologies) and stored at -80°C. For each sample, 2 μL were analysed. A 260/280 nm and 260/230 nm absorbance ratio of approximately 2.0 or 2.0-2.2 respectively was an indicator of pure RNA.

2.6.1 RTPCR

All primers were custom designed and synthesized (Sigma, UK) (see table 2-3) [171]. Amplifications were performed in 25 μL reactions using ExTaq polymerase buffer (Gibco, UK) and 1 μM of each primer as follows - initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 15 sec; primer-specific annealing for 30 sec; extension for 1.5 to 2 minutes based on amplicon length and a final extension at 72°C for 10 min. PCR products were resolved on 2% agarose gels containing 0.5 mg/mL ethidium bromide. Images were visualized using a UV

transilluminator. Sequencing was also performed by the protein nucleic acid chemistry laboratory (PNACL), University of Leicester. This work was carried out by Dr Emma Parrott (Post-doctoral Researcher, Leicester Cancer Research Centre, University of Leicester).

Table 2-3. Overview of custom designed TaqMan probes.

Target gene	Forward primer	Reverse primer	Amplicon size
Nanog 387	TGTCTTCTGCTGAGATGCCTCACA	CCTTCTGCGTCACACCATTGCTAT	387
Nanog 1860	TGAGTGTGGATCCAGCTTGT	TCATCGAAACACTCGGTGAA	1860

2.7 Cignal Reporter Assay

Caco-2 cells were passaged 1:3, 24 hours prior to plating assay as described above, thereby ensuring they were not over confluent. Cells were allowed to adhere and then harvested and resuspended to 2 x 10^5 cells/mL in OptiMEM media supplemented with 5% FCS and 1% non-essential amino acids (all Gibco, UK). 20000 caco-2 cells were added to each well, in a 96 well plate. Briefly, OptiMEM media, Lipofectamine (Invitrogen, UK) and PLUS reagent (Invitrogen, UK) were incubated for 5 minutes. To this, Nanog reporter DNA (Qiagen, UK) was added and incubated for a further 20 minutes. Regents were added at the following ratios: DNA: Lipofectamine 1:2.5. Plus reagent was added at a ratio of 1 μL per 1 μg of DNA. Subsequently 20000 caco-2 cells were plated with the DNA cocktail in flat-bottomed white luminescent plates (Perkin Elmer, UK). Cell medium was carefully changed following 24 hours of incubation ensuring cells were not disturbed. The following day, cells were treated with curcumin (0-1 μM) for 24 hours.

To prepare cells for measuring luminescence, all media was removed from cells, cells were washed in PBS and passive lysis buffer added for 20 minutes on a rotating shaker. To develop the assay, luciferase assay buffer was added to the cells, firefly activity measured for 12 seconds using a luminescence measure, stop and glo reagent added to quench the signal, and renilla activity measured for 12 seconds (Dual Luciferase

Reporter system, Promega, UK) using a Fluostar Optima plate reader. Promoter activity values were expressed as a ratio using the renilla reporter for internal normalisation. Results are reported as a percentage of control ratio. Over this 5 day period, the confluency of cells was carefully monitored to ensure optimum transfection and signal read out.

2.8 Western blotting analysis

2.8.1 Production of cell lysates

Caco-2 and HCT116 cells were treated and then harvested as described previously. However, ice cold PBS was used to wash the cells and cells were kept on ice throughout processing. Radioimmunoprecipitation assay (RIPA) buffer (Sigma, UK) supplemented with 1 tablet of phosphatase inhibitor and 1 tablet of complete mini (Roche, UK) was added to cell pellets in a 1:1 weight to volume ratio. Cells were kept on ice for 30 minutes, centrifuged at 1600 rpm (200 *x* g) for 10 minutes at 4°C, supernatant collected and stored at -80°C until required.

2.8.2 Bicinchoninic acid assay (BCA)

The BCA was performed to quantify soluble protein in lysates (Thermoscientific Pierce, UK). A bovine serum albumin (BSA) standard curve was prepared using 2 mg/mL and diluted as listed in table 2-4.

Table 2-4. Preparation of BSA standard curve for BCA

BSA standard concentration	Volume of double distilled water	Volume of BSA
1 mg/mL	250 μL	250 μL of 2 mg/mL BSA stock (provided by kit)
0.8 mg/mL	20 μL	80 μL of 1 mg/mL
0.6 mg/mL	40 μL	60 μL of 1 mg/mL
0.4 mg/mL	60 μL	40 μL of 1 mg/mL
0.2 mg/mL	80 μL	20 μL of 1 mg/mL
0 mg/mL	100 μL	0 μL of 1 mg/mL

Abbreviations: Bovine serum albumin (BSA), Bicinchoninic acid assay (BCA)

Each protein sample was diluted 1 in 10, 1 in 20 and 1 in 100 using double distilled water. When using sorted-cell lysates, each protein sample was diluted 1 in 20 and tested in triplicate. The BSA standard and each protein sample was pipetted into a 96 well place in triplicate. Following plating of samples, 200 μL of BCA reagent was added to all wells and incubated at 37°C for 30 minutes. Subsequently, samples were scanned at 595 nm wavelength using a Fluostar Optima plate reader (LICOR, UK) and protein concentrations were determined from the BSA standard curve using the formula $y=mx+c$.

2.8.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For all proteins investigated either an 8% or 10% gel was used. A gel casting apparatus was set up according to manufacturer's instructions (Biorad, mini gel apparatus). Resolving gels were prepared at 5%. The resolving gel was made first, allowed to set and the stacking buffer added with a 10 or 15 well comb inserted. Once set, the comb was removed, and the gel cassette placed in a running tank with 1x Tris/Glycine/SDS running buffer prior to proteins being loaded (see table 2-5).

Table 2-5. Outline of reagents needed for resolving and stacking gels.

Reagent	Resolving gel 8%	Resolving gel 10%	5% Stacking gel
Double distilled water	9.5 mL	8.1 mL	5.7 mL
Protogel resolving or stacking buffer (Geneflow, UK)	5 mL	5 mL	2.5 mL
Protogel, 30% Acrylamide (Geneflow, UK)	5.3 mL	6.7 mL	1.7 mL
TEMED (Sigma, UK)	15 μL	15 μL	15 μL
10% APS (Sigma, UK)	0.2 mL	0.2 mL	0.1 mL

Abbreviations: Tetramethylethylenediamine (TEMED), Ammonium persulfate (APS)

Reagents required for SDS- PAGE

Running buffer: made by diluting 100 mL 0.25M Tris/1.92M Glycine/1% SDS (Geneflow UK) with 900 mL double distilled water.

Transfer buffer: made by diluting 100 mL of 0.25M Tris/1.92M Glycine (Geneflow, UK) with 700 mL double distilled water and 200 mL methanol.

Ammonium persulfate (APS) (Sigma, UK): prepared by weighing 1 g in 10 mL water (10%, w/v ratio).

Phosphate buffered saline Tween 20 (PBST20) 0.1%: prepared by dissolving 10 tablets of PBS (Oxoid, UK) in 1000mL distilled water with 1 mL Tween 20 (Sigma, UK).

Blocking buffer: prepared with 2.5g milk (Marvel, UK) in PBST20 i.e. 5% milk. Antibodies were diluted in 3% milk.

2.8.4 Running and transferring of protein samples

Protein samples were defrosted on ice, and appropriate volumes of protein diluted in buffer to provide samples of equivalent concentrations in a final volume of 10 μL. An equal volume of loading buffer (x2 Laemmli, Sigma UK) was added to protein lysate,

making a total volume of 20 μL. Samples were then heated at 95°C for 5 minutes allowing denaturation to occur. Samples underwent a pulse spin and were loaded into wells to run at 120 mv for 45 minutes at room temperature.

Proteins were transferred from the gel onto a Hybond ECL nitrocellulose membrane (0.2 μM) as follows; gels, Hybond ECL nitrocellulose membrane, Whatmann paper and sponges were pre-soaked in transfer buffer x1, gels were places into a transfer cassette which consisted of x1 sponge, Whatmann paper (Sigma, UK), gel, nitrocellulose membrane (Geneflow, UK), Whatmann paper and x1 sponges. The transfer cassette was placed in a transfer tank with transfer buffer. Protein transfer was undertaken at 100 V for 90 minutes at room temperature.

2.8.5 Blocking and antibody probing

Following transfer of proteins onto Hybond ECL nitrocellulose membrane, the membrane was blocked in 5% milk overnight. The nitrocellulose membrane was then washed in PBS Tween 20 (PBST20 0.1%) for 3 separate 10 minute washes. The primary antibody was prepared in 3% milk in PBST. Dilutions were decided upon following optimisation (see table 2-6 and 2-7). The membrane was incubated with primary antibody diluted in 3% milk at room temperature for 1 hour. Subsequently the membrane was washed and secondary antibody added for 1 hour at room temperature. Lastly, the membrane was washed prior to detection of proteins.

Table 2-6. Primary antibody dilutions

Primary antibody	Primary antibody dilution
Nanog (Novus Biologicals, UK)	1:500
PhosphoNanog (Thermoscientific, UK)	1:500
Nanog (R&D, UK)	1:500
Oct4 (Novus Biologicals, UK)	1:1000
BMI 1 (Abcam, UK)	1:500
FAK (Cell Signaling Technology, UK)	1:500
Actin (Santa Cruz, UK)	1:1000

Abbreviations: Octamer-binding transcription factor 4 (Oct4), B Lymphoma Mo-MLV insertion region 1 homolog (BMI 1), Focal adhesion kinase (FAK).

Table 2-7. Secondary antibody dilutions

Primary antibody	Secondary antibody	Secondary antibody dilution
Nanog (Novus Biologicals, UK) PhosphoNanog (Thermoscientific, UK) Nanog (R&D, UK)	Anti- Rabbit HRP linked antibody (Cell Signaling Technologies, UK) Anti-Goat HRP linked antibody (Santa Cruz, UK)	1:5000 1:1000 1:1000
Oct4 (Novus Biologicals, UK)	Anti- Rabbit HRP linked antibody (Cell Signaling Technologies, UK)	1:2000
BMI 1 (Abcam, UK)	Anti- Rabbit HRP linked antibody (Cell Signaling Technologies, UK)	1:2000
FAK (Cell Signaling Technology, UK)	Anti- Rabbit HRP linked antibody (Cell Signaling Technologies, UK)	1:2000
Actin (Santa Cruz, UK)	Anti-Goat HRP linked antibody (Santa Cruz, UK)	1:10000

Abbreviations: Octamer-binding transcription factor 4 (Oct4), B Lymphoma Mo-MLV insertion region 1 homolog (BMI 1), Focal adhesion kinase (FAK).

2.8.6 Detection of proteins

Following the final wash, the Hybond nitrocellulose membrane was placed in enhanced chemiluminescence (ECL) solution (Geneflow, UK) for 5 minutes. The ECL solution was prepared using a 1:1 ratio of reagents A and B, incubated for 5 minutes at room temperature (Thermoscientific, UK). Once the membrane had been incubated with ECL, the excess solution was removed, the membrane wrapped in cling film and put into an X-Ray developing hypercassette (Amersham, UK). In a dark room, the membrane was

exposed to X-Ray film for 10 seconds to 1 minute and the film developed using an AGFA CURIX 60 automated developer (AGFA Gevaert, Germany) or following cling film the membrane was placed in a Syngene developer (Syngene, UK). Image J or Syngene software was used for analysis.

2.9 Gene expression analysis – Nanog, BMI1 and FAK

2.9.1 RNA extraction, elimination of genomic DNA and synthesis of first stand cDNA

Total RNA was isolated from cell pellets, genomic DNA contamination removed and RNA quantified as previously described. cDNA was synthesised from extracted RNA using a QuantiTect Reverse Transcription Kit (Qiagen, UK) as per manufacturers' instructions.

2.9.2 Real time quantitative reverse transcription polymerase chain reaction (RTqPCR)

RTqPCR reactions were performed using an ABI StepOne Plus RTqPCR machine. Each reaction was made with 4.5 μL cDNA, 5μL TaqMan Fast Universal Master Mix (2x) (Life Technologies, UK) and 0.5 μL target specific TaqMan probe (20x) (Life Technologies, UK) (see table 2-8). Each reaction was added to a MicroAmp Fast Optical 96 well PCR reaction plate (Applied Biosystems, UK) with an adhesive covers (Applied Biosystems, UK). The short cycle parameters were 95°C at 40 seconds, plus 40 cycles of 95°C at 1 second and 60°C at 20 seconds.

Table 2-8. Overview of TaqMan probes.

Target gene	Amplicon length (base pairs)
Nanog	121
BMI 1	76
FAK	84
GAPDH	58
Actin	171

Abbreviations: Octamer-binding transcription factor 4 (Oct4), B Lymphoma Mo-MLV insertion region 1 homolog (BMI 1), Focal adhesion kinase (FAK), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.10 Cycloheximide (CHX) and emetine experiments

A CHX assay was carried out using HCT116 cell lines to investigate the effect of curcumin on Nanog protein turnover. Cells were seeded at a density of $0.5x10^6$ per flask/plate for 24 hours, prior to treatment with 0.1 μM curcumin and either CHX or emetine. Cells were harvested at 10/20/30/60 minutes and 2/4/6 hours as described previously. RIPA buffer supplemented with 1 tablet of phosphatase inhibitor and 1 tablet of complete mini was added to cell pellets in a 1:1 weight to volume ratio. Cells were kept on ice for 30 minutes, centrifuged at 1600 rpm (200 *x* g) for 10 minutes at 4°C, supernatant collected and stored at -80°C. Nanog expression was quantified using western blotting. Actin was used to normalise data.

2.11 Secondary sphere experiments

To investigate the effect of long-term low dose curcumin treatment on CSC resistance, HCT116 spheres were plated as described (1000 cells/well were plated). Cells were treated on alternate days for 17 days with curcumin (0-10 μM). Spheres were harvested, counted and replated (1000 cells/well). Cells were monitored for sphere formation for 70 days. At this point, spheres had grown in all wells. Cells were retreated on alternate days for 5 days and sphere growth monitored. Cells were monitored for sphere formation for a further 30 days and retreated. Prior to each retreatment of cells, images were taken

using a LICOR DMi8 microscope. Leica application suite X software was used to obtain images.

2.12 Statistical analysis

Statistics were carried out using Graph Pad Prism or SPSS version 20. Data was tested for normality and either parametric or non-parametric tests were used. The null hypothesis was rejected when any p-value was equal to or below 0.05 between parameters and considered to be statistically significant.

3.0 Chapter 3 (Results and Discussion) - CSC marker expression in CRC tissues

3.1 Introduction

Baseline expression of CSC markers in primary CRC tissues have been published over recent years [172-180]. This is often investigated in patient tissues using IHC or gene expression analysis. Many cohorts are small with limited information on progression-free survival in response to treatment and overall survival in relation to CSC markers. In addition, the feasibility of being able to target these markers is not yet available. Based on unpublished data within the group, CSC markers ($Nanog^+$, $Nanog^+Ki67^+$ and aldefluor vs $ALDH1A1^+$ and $Nanog^+ALDH1A1^+$) were tested across a number of normal, adenoma and cancer tissues and across different stages of cancer and paired samples. Some of these samples were used in explant assays to assess efficacy of curcumin treatment. In addition, in these samples CMS subtypes have been considered. The work regarding CMS subtypes is still ongoing. Of note, normal samples were obtained from patients who were undergoing resections for CRC, but taken from macroscopically normal mucosa at least 10cm away from the tumour.

Knowledge of baseline expression in primary samples allows information gained in cell lines to be translated back to a clinical setting. Data here will also be useful when designing clinical trials and allow the feasibility of using certain CSC markers as an indication of treatment efficacy to be established. Most data regarding Nanog and patient outcome is based on IHC data [181-183]. There are no data currently available assessing Nanog expression using a flow cytometry based assay correlated with patient outcomes. It is hoped when the data presented here matures, this can be assessed (currently <5 patients have died following treatment). There is scarce data on adenoma samples in the literature. The advent of organoid culture has allowed this to be more systemically investigated in terms of transcriptomic and proteomic expression, which will aid molecular understanding of cancer in patients i.e. similar processes and diverse expression are recapitulated in organoids [184-186].

3.2 Profiling of cancer stem-like cell markers in primary colon tissues

3.2.1 Marker profiling in normal, adenoma and cancer tissues

To investigate the levels and variation in cancer stem-like markers in human colorectal samples, single cells obtained from primary adenomas and cancers, along with matched normal tissue where available were stained and analysed using flow cytometry. Nanog, ALDH1A1 and Ki67 expression was analysed.

3.2.2 Demographics

Demographics of the patient samples used for intracellular staining are shown in table 3-1. There is a higher proportion of male compared to female samples in this cohort (29 samples vs 17 samples). This reflects the incidence of CRC in the UK (1 in 14 men and 1 in 19 women will be affected by CRC in their lifetimes). The average age of sample participants was approximately 69 years for both men and women [7]. This is representative of the UK population affected by CRC In terms of stage of cancer, a slightly higher proportion of stage 2 and 3 cancers has been collected. In total 37 stage 2 and 3 samples were collected versus 9 stage 1 and 4 samples. This is probably due to a multitude of factors including, ease of access to these samples e.g. stage 1 cancers, may undergo limited resections and have less 'excess' tissue available without compromising clinical care or stage 4 cancer patients not undergoing surgery. Some stage 4 patients may undergo surgery due to complications e.g. obstruction or perforation or cancers have been 'down staged' following chemotherapy allowing resection. In addition, there is no pathway established for collection of tissues out of normal working hours. It is worth noting that individuals being treated for stage 2 and 3 cancers may be offered adjuvant chemotherapy and may also benefit from alternative therapeutic prevention opportunities so would be an ideal cohort to consider sampling. Nationally, a higher proportion of left sided cancers are diagnosed than right sided cancers which is reflected in the patient samples analysed. In total 25 left and 21 right sided samples were collected. In right sided and rectal cancers, 32% men and 23% women are affected. However, in sigmoid cancers the demographics are 23% men and 20% women. In contrast, in left sided cancers e.g. the caecum, only 12% men and 17% women are affected and for the ascending colon 7% men and 10% women are affected [141]. In summary, a reasonable sized cohort (46 cancer samples and 6 adenoma samples) were analysed which are reflective of the UK CRC burden.

Table 3-1. Demographics of samples used for intracellular staining (Nanog$^+$/ALDH1A1$^+$ and Ki67$^+$ expression).

Gender	Male	Female
n	29	17
Mean age at diagnosis	68.6 years	69.6 years
Range	46-92 years	35-89 years
Cancer Stage		
1	5	1
2	8	8
3	14	7
4	2	1
Site of cancer		
Left sided cancer	20	5
Right sided cancer	9	12
Adenomas	3	3

3.2.3 Gating strategy and example plots

Tissues were processed into single cells, fixed and permeabilised for intracellular staining. For each marker of interest, the unstained population was used to help gate for positive cells (an isotype was used for Nanog staining due to concerns regarding non-specific staining during optimisation experiments and a single peak for positive cells was not detected). Using these populations of cells, double positive cells were identified (see figure 3-1). Single replicates were used due to limitations in cell number obtained from primary samples e.g. <1 g of tissue obtained and necrotic samples limiting viability of cells.

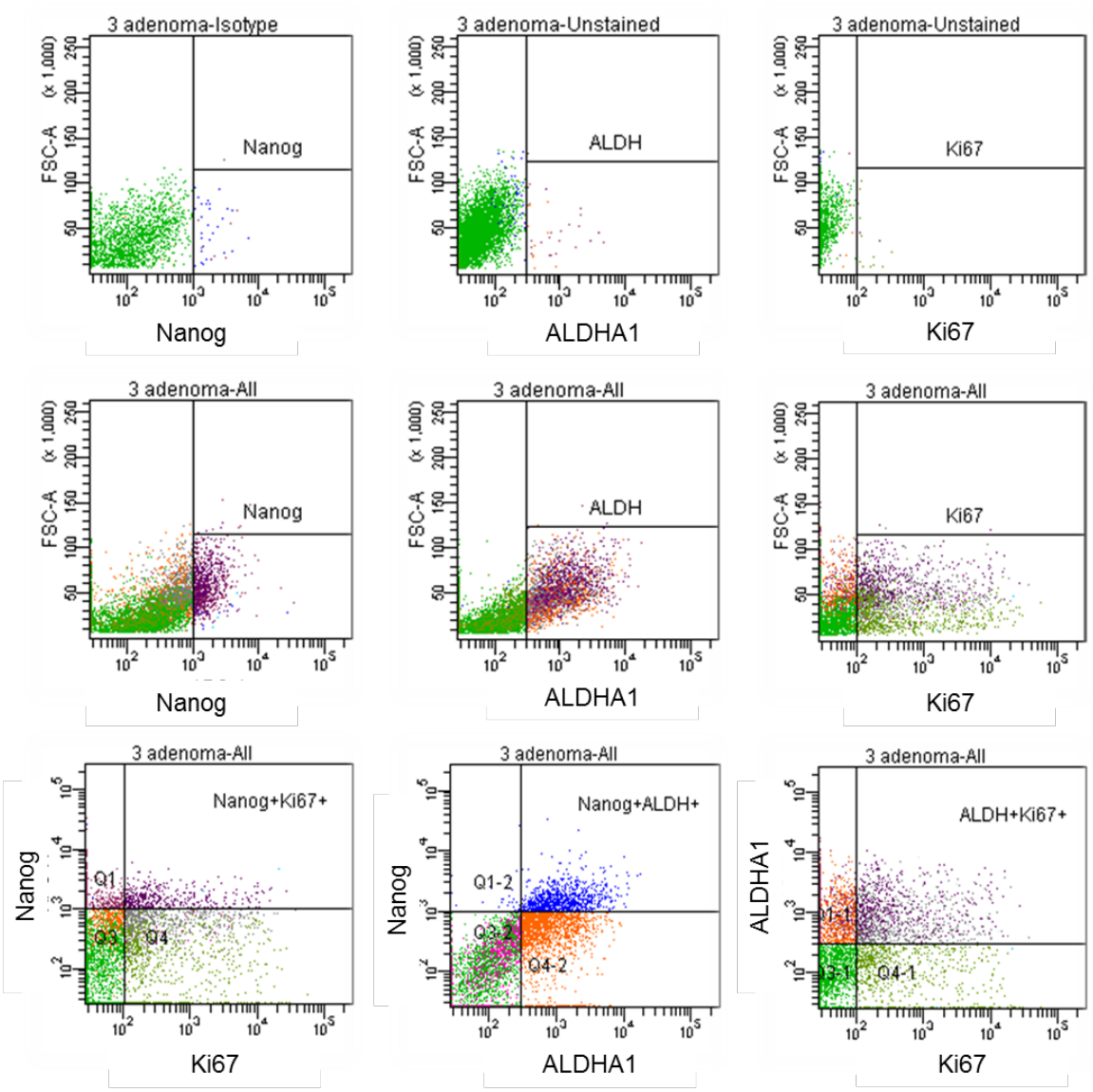


Figure 3-1. Example plots and gating strategy used to identify Nanog$^+$, ALDH1A1$^+$, Ki67$^+$, Nanog$^+$Ki67$^+$, Nanog$^+$ALDH1A1$^+$ and ALDH$^+$Ki67$^+$ cells. Single cells were fixed and permeabilised, antibodies added and analysis carried out. Unstained populations or Nanog isotype are shown (top row). These gates were used to identify single positive populations i.e. Nanog$^+$, ALDH1A1$^+$ and Ki67$^+$ populations (middle row). Lastly, using bivariate plots double positive i.e. Nanog$^+$Ki67$^+$, Nanog$^+$ALDH1A1$^+$ and ALDH1A1$^+$Ki67$^+$ populations were calculated. Here an adenoma sample is used as representative of all samples analysed. Analysis was carried out in single replicates, with known positive and negative controls (caco-2 cell line).

3.2.4 Nanog$^+$ expression in colorectal tissues

Significantly higher levels of Nanog$^+$ were identified in adenoma samples (6.85%) and cancer samples (3.47%) compared to normal controls (0.88%) (Figure 3-2). Low levels of Nanog$^+$ was detected in normal tissues. It is notable that higher levels of Nanog$^+$ are detected in adenoma tissues. The rationale for this is unclear, it may be due to smaller sample size (i.e. 6 samples), reflecting the greater variance in samples described. Consideration may also be given to Nanog$^+$ being a driver of carcinogenesis in colorectal cancer. In addition, there has been data suggesting that Nanog positivity is linked with poorer outcomes in CRC, therefore being a marker of poor prognosis. Taken together, tis data may demonstrate that Nanog may be a potential marker, which could be targeted in CRC prevention and treatment [182, 183].

When considering paired normal and cancer tissue samples i.e. both normal sample and cancer sample from the same patient (36 patient samples), cancer samples (3.83%) had significantly higher levels of Nanog$^+$ compared to normal tissues (0.91%). However, when considering adenoma samples (3 patient samples with mean value 10.2%) to paired normal tissues (0.43%), this was not significant. This is likely due to small sample and size and large variation in Nanog$^+$ expression as far higher levels of Nanog$^+$ were detected in adenoma samples compared to normal or cancer tissues.

No difference in expression was detected between left and right sided cancers or male and female cancers (data not shown).

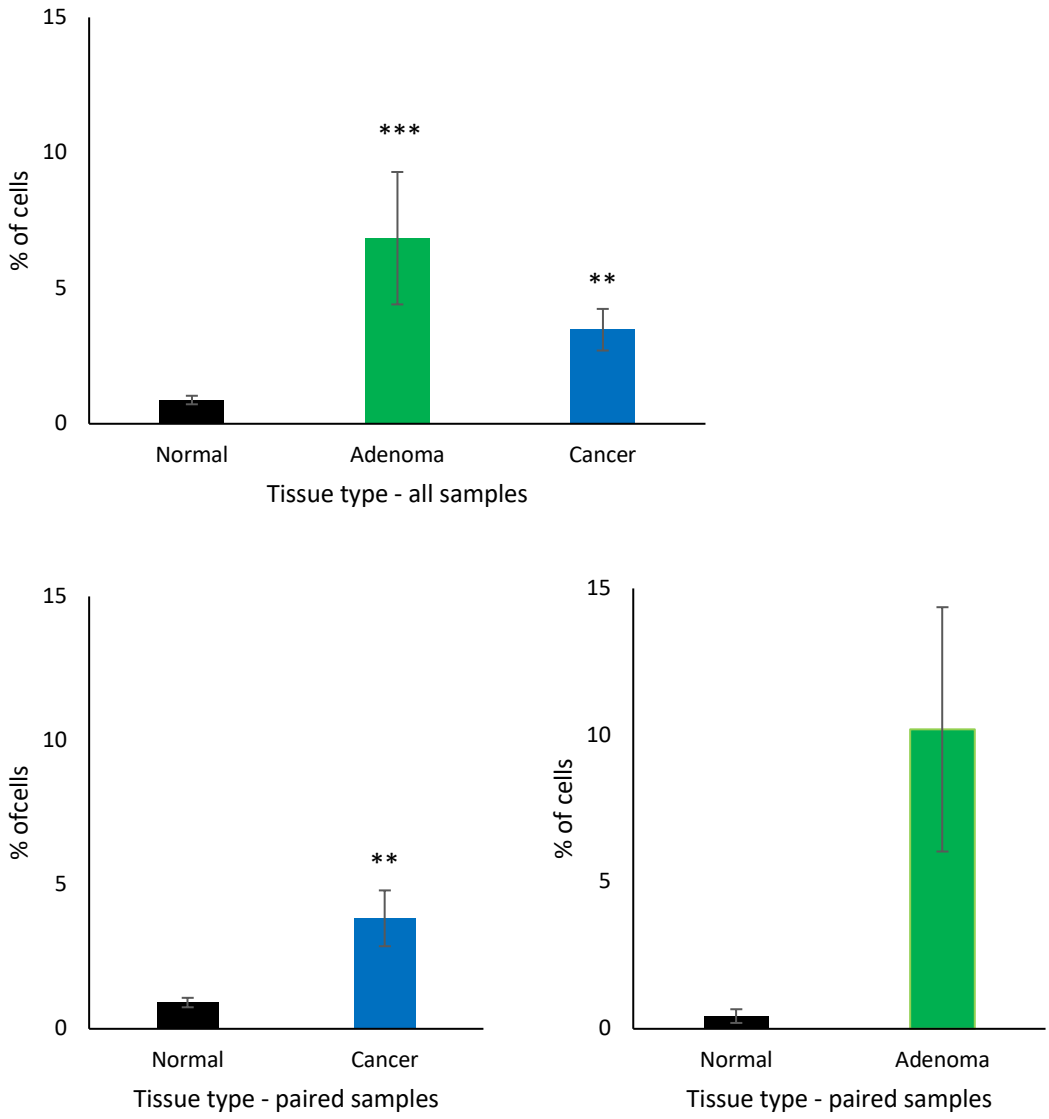


Figure 3-2. Baseline Nanog$^+$ expression in normal, adenoma and cancer tissues. Single cells from primary colon samples were assessed for Nanog$^+$ expression using intracellular staining. All values represent mean ± SEM, where n = 38 normal, 46 cancer and 6 adenoma samples. 36 cancer samples had a matched normal and 3 adenoma samples had a matched normal. Non-significant changes were detected between adenoma and cancer samples (***P<0.001, **P<0.01 as determined by 2-way ANOVA test).

3.2.5 Nanog$^+$Ki67$^+$ expression in colorectal tissues

Significantly higher levels of Nanog$^+$Ki67$^+$ were identified in adenoma samples (4.60%) and cancer samples (2.18%) compared to normal controls (0.39%) (see Figure 3-3). Low levels of Nanog$^+$Ki67$^+$ were detected in normal tissues. Higher levels of Nanog$^+$Ki67$^+$ are detected in adenoma tissues. Over half of the total population of Nanog$^+$ cells is proliferating (6.85% total Nanog$^+$ population, with proliferating proportion 4.6%) in adenoma tissues and cancer tissues (3.47% and 2.19%) suggesting this fraction of cells is actively dividing and may be targetable by therapies in an early or later stage of cancer prevention or treatment.

No difference in expression was detected between left and right sided cancers or male and female cancers (data not shown).

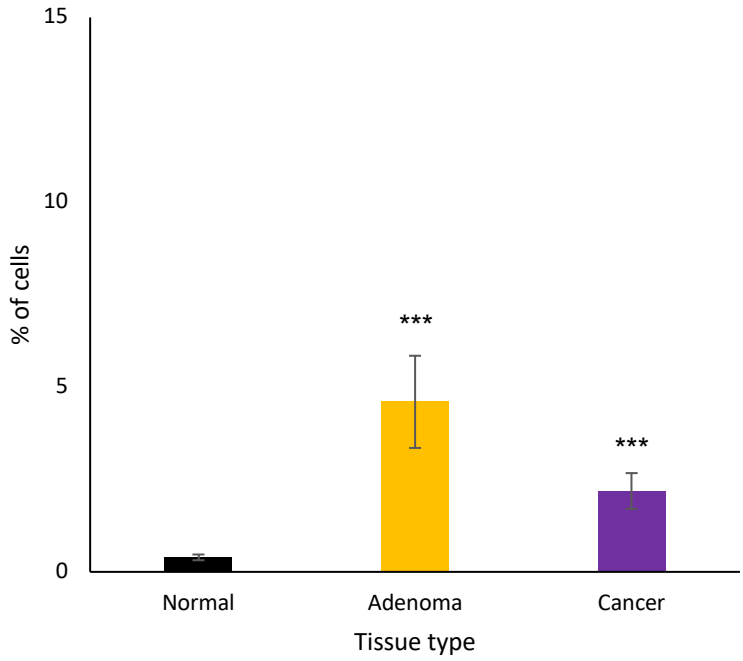


Figure 3-3. Baseline Nanog$^+$Ki67$^+$ expression in normal, adenoma and cancer tissues. Single cells from primary colon samples were assessed for Nanog$^+$KI67$^+$ expression using intracellular staining. No significant changes were detected between adenoma and cancer samples. All values represent mean ± SEM, where n = 38 normal, 46 cancer and 6 adenoma samples (***P<0.001, **P<0.01 as determined by as determined by 2-way ANOVA test).

3.2.6 ALDH1A1^{+} and aldefluor staining comparison

The difference between ALDH activity and ALDH1A1^{+} expression has been considered in the background to this work. To investigate whether this had an impact on CRC samples analysed, the same patient samples were subjected to ALDH activity and ALDH1A1^{+} expression testing.

3.2.6.1 Demographics

Patient demographics are listed (see table 3-2). These were broadly representative of the UK population affected with CRC, however, no female left sided or early stage female cancers were analysed.

Table 3-2. Demographics of samples used for ALDH1A1^{+} expression and ALDH activity.

Gender	Male	Female
n	12	8
Mean age at diagnosis	66.0 years	74.6 years
Range	46-92 years	45-88 years
Cancer Stage		
1	3	0
2	2	3
3	6	4
4	1	1
Site of cancer		
Left sided cancer	7	0
Right sided cancer	5	8

3.2.6.2 Gating strategy and example plots

Tissues were processed into single cells, and staining carried out using aldefluor, CD133 and ESA. For each marker of interest, the unstained population was used to help gate for positive cells (for aldefluor a negative control was used as described previously). Using these populations of cells, double positive cells were identified [187]. Single replicates were used due to limitations in cell number obtained from primary samples e.g. <1g of tissue obtained and necrotic samples limiting viability of cells.

No significant difference in ALDH activity were observed between normal or cancerous tissues (3.72% and 3.44% respectively). ALDH1A1 expression was higher in normal tissues versus cancerous tissues (12.9% vs 9.08%), although this difference was non-significant (figure 3-4). This potentially could be due to cell viability. As the ALDH activity requires live/viable cells, these may have been compromised during freeze/thawing however apoptotic and necrotic cells were not specifically assessed in this assay. Additionally, this is possibly due to an additional marker used to establish epithelial cells in the ALDH activity assay (ESA^+) so these cells are ESA^+ALDH^{high}. Whether to use an epithelial marker is contentious. As more is known regarding the plasticity of CSCs, it may be that cells which do not express ESA harbour CSC properties i.e. they may be undergoing EMT [188] [189]. Importantly, it may be due to the non-specific nature of quantifying $ALDH1A1^+$ expression vs ALDH activity, highlighting difficulties in correlating ALDH activity with $ALDH1A1^+$ expression in samples.

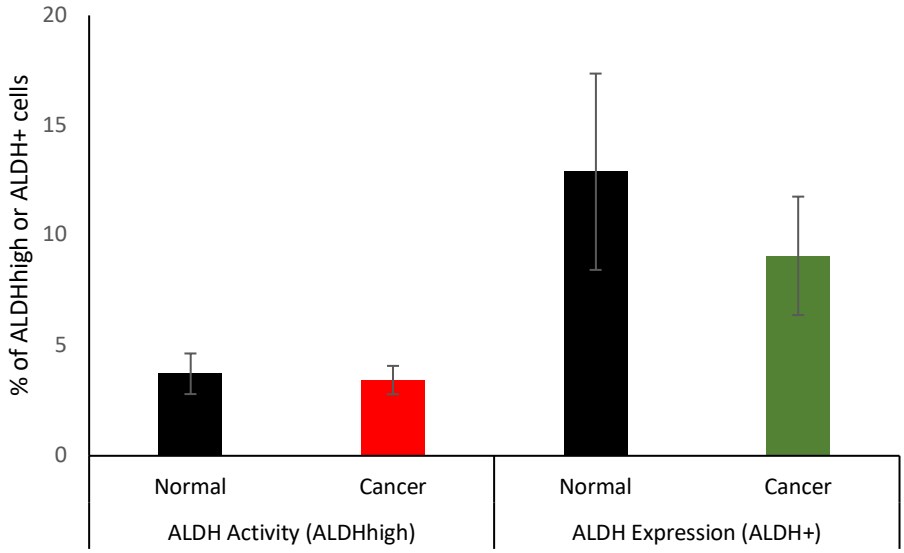


Figure 3-4. Baseline ALDH^{high} and ALDH1A1^{+} expression in normal and cancer tissues. Single cells from primary colon samples were assessed for ALDH^{high} and ALDH1A1^{+} tissues. All values represent mean±SEM, where n=20 samples. Data obtained from ALDH^{high} cells was similar to those which were ESA^{+}ALDH^{high} (data not shown).

3.2.7 Nanog^{+}ALDH1A1^{+} expression in colorectal tissues

Significantly higher levels of Nanog^{+}ALDH1A1^{+} were identified in adenoma samples (5.56%) and cancer samples (2.35%) compared to normal controls (0.55%) (see Figure 3-5). Low levels of Nanog^{+}ALDH1A1^{+} were detected in normal tissues. Higher levels of Nanog^{+}ALDH1A1^{+} are detected in adenoma tissues. As Nanog^{+} appears to be a subpopulation of ALDH1A1^{+} cells, this may explain the correlation between Nanog^{+}, Nanog^{+}Ki67^{+} and Nanong^{+}ALDH1A1^{+} markers. It is unclear of the significance of this biologically or clinically.

No difference in expression was detected between left and right sided cancers or male and female cancers (data not shown).

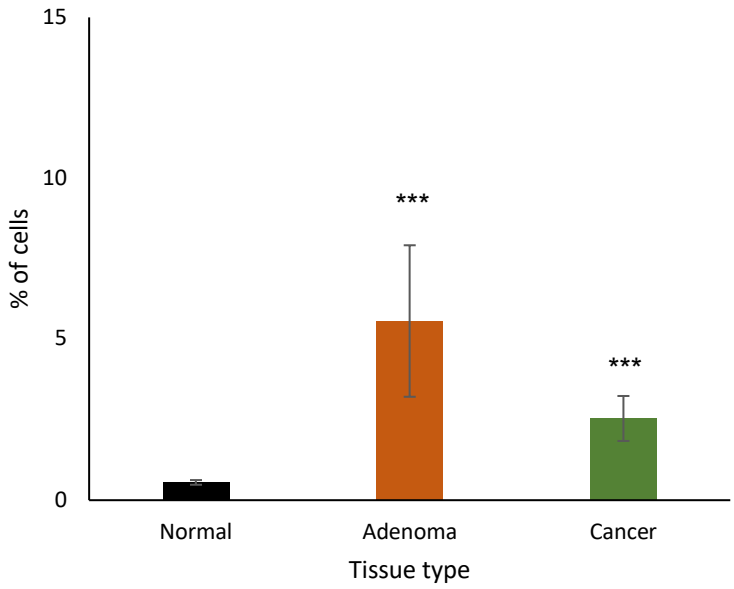


Figure 3-5. Baseline Nanog$^+$ALDH1A1$^+$ expression in normal, adenoma and cancer tissues. Single cells from primary colon samples were assessed for Nanog$^+$ALDH1A1$^+$ expression using intracellular staining. All values represent mean ± SEM, where n=38 normal, 46 cancer and 6 adenoma samples (***P<0.001, **P<0.01 as determined by student's T Test).

3.2.8 Nanog$^+$ and Nanog$^+$Ki67$^+$ expression across colorectal cancer stages

Both Nanog$^+$ and Nanog$^+$Ki67$^+$ expression increases over stage 1-3 cancers and then drops at stage 4. Levels are significantly higher than in normal tissues and highest in adenoma tissues as previously described. This indicates that a Nanog targeting cancer prevention or treatment agent could potentially be useful in a premalignant setting and all stages of cancer treatment (Figure 3-6 and 3-7).

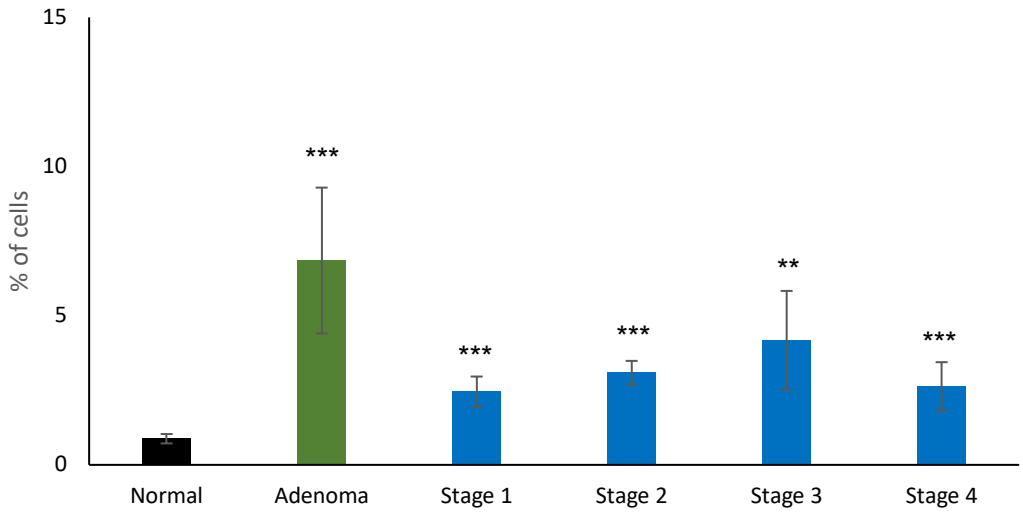


Figure 3-6. Baseline Nanog$^+$ expression in normal, adenoma and cancer tissues. Single cells from primary colon samples were assessed for Nanog$^+$ expression using intracellular staining. All values represent mean±SEM, where n=38 normal, 46 cancer and 6 adenoma samples (***P<0.001, **P<0.01 as determined by student's T Test).

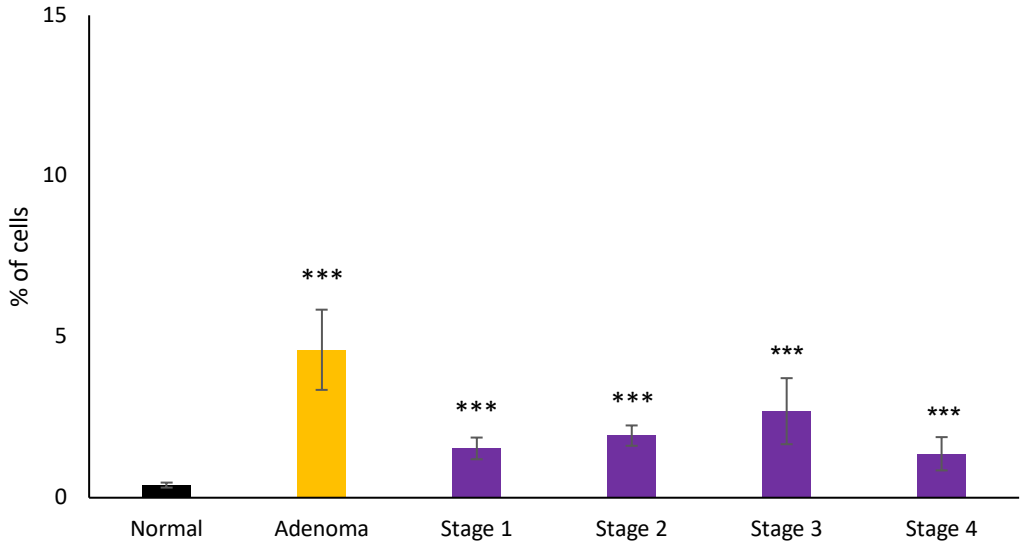


Figure 3-7. Baseline Nanog$^+$Ki67$^+$ expression in normal, adenoma and cancer tissues. Single cells from primary colon samples were assessed for Nanog$^+$Ki67$^+$ expression using intracellular staining. All values represent mean±SEM, where n=38 normal, 46 cancer and 6 adenoma samples (***P<0.001, **P<0.01 as determined by student's T Test).

3.3 Conclusion

The identification of CSCs has caused controversy for many years [131, 190-192]. Initially, the hallmarks of a CSC population were thought to be characterised by a specific set of markers that associate with the following attributes: resistance to standard treatments; able to grow in serum-free conditions in culture; able to grow in immunocompromised mice [193]. Over time, this has been challenged, particularly in solid tumours. CSC populations involving haematological tumours and embryonic stem cells have a greater consensus on markers and properties compared to solid tumours. The heterogeneity in solid tumours results in the identification of CSCs to be difficult. In addition, the method by which they are derived such as intracellular staining or surface marker staining affects functionality. For example intracellular staining renders the cell dead, therefore subsequent experiments such as growth in an immunocompromised mouse cannot be carried out. This results in a skew in knowledge regarding these markers (such as Nanog) as functional assays are difficult to carry out. Despite this, Nanog is also an embryonic stem cell marker and has been linked with stem features and sphere forming capacity in a number of cancers and associated pre-clinical models [194]. In addition, there is limited data regarding the protein structure of Nanog and proteins which may bind to it e.g. Oct4 and DNA-binding elements as described previously.

The detection of CSC markers in human tissues and resulting effects on outcomes is evidenced in the literature [172-174]. CSC markers e.g. $LgR5^{+}$, $ALDH1A1^{+}$ and Nanog are associated with poor patient outcomes. Often markers are detected by IHC and grouped in to low/medium and high expression rather than using a method which is able to produce a numerical output of quantification. With regards to pre-malignant tissue, far less data is available [195, 196]. The data presented here, suggests higher levels of $Nanog^{+}$ expression are detectable in cancer tissues and adenoma tissues compared to normal tissues. The highest levels of Nanog were detected in adenoma tissues. It is unclear, the reasons behind such high levels, however, it would seem reasonable to hypothesise that a reduction in $Nanog^{+}$ expression in adenoma tissues may result in a slowing down of progression to malignant tissues.

4.0 Chapter 4 (Results and Discussion) – Effect of curcumin on modulation of cancer stem-like cells using caco-2 cells

4.1 Introduction

Unpublished data from our group using primary spheroids and xenografts derived from patient CRC CSCs (defined as $ALDH^{high}$) supports the hypothesis that curcumin is able to target CRC CSCs [187, 197]. The exact mechanism of this is unclear. The primary aim of this chapter of work is to characterise this mechanism further using immortalised colorectal cancer cell lines in 2D and 3D culture. Caco-2 cells (unsorted cells and sorted cells defined by $ALDH^{high}$) were used to consider the effects of curcumin on transcriptional activity and protein expression of CSC markers (Nanog and Oct4) and downstream targets of Nanog. This work was further validated using a genetically modified cell line, $HCT116^{GFP/Nanog}$. This cell line has been designed to overexpress Nanog protein with a matched control cell line ($HCT116^{GFP}$). These cells were cultured as 3D spheres. This will allow potential markers of biological activity and mechanism to be validated further in human 3D explant cultures and RNASeq experiments in the future.

The advantages of using 3D models instead of 2D have been documented extensively. Specifically, though 2D culture offers, a simple, fast and cost effective method to study pharmacological agents, they do not fully recapitulate the 3D environment they are intended for e.g. immune cells, extracellular matrix and a heterogeneous population of cells. In addition, signal transduction pathways and absorption of drugs are difficult to assess in a 2D setting. There remain limitations using 3D models e.g. diversity of cells make it difficult to replicate experiments, and they are expensive to carry out in large scale experiments/screening methodologies. Importantly, methods to take into account vasculature are limited [197-199].

As well as using 3D cultures, where practically feasible, it is important to consider the molecular subtyping of cell lines used. Caco-2 cells are microsatellite stable, and wild type for key mutations in colorectal cancer (BRAF, PIK3CA, PTEN and KRAS) [170]. In contrast, HCT116 cells, are microsatellite unstable, and contain mutations in KRAS and PIK3CA [170]. In terms of CMS, HCT116 are thought to fall within CMS1, whereas Caco-2 cells are CMS4 [200]. Though a consensus amongst different groups has not been reached. Ideally, each CMS subtype and mutation should be considered, however, this

represents a reasonable range for preliminary mechanism and biological activity to be identified, which can be further validated in human 3D explant cultures.

4.2 Effect of curcumin on transcriptional activity of Nanog protein in Caco-2 cells (2D)

As Nanog is a transcriptional factor and homeodomain containing protein, Nanog transcriptional activity was assessed in the presence of curcumin. Curcumin significantly decreased the transcriptional activity of Nanog at 0.1 and 1 μM curcumin treatment to 51% and 53% respectively (see figure 4-1). This may be due to decreased binding of Nanog to the DNA response element, thereby reducing the ability of Nanog to maintain the CSC phenotype. In addition, curcumin affinity pull down assays have demonstrated binding of curcumin to the homeodomain portion of Nanog protein (figure 4-2). The effect of curcumin on Nanog-DNA interaction was further evaluated using the electrophoretic mobility shift assays. Here curcumin had a dose dependent effect on reducing Nanog-DNA interaction (figure 4-3) [201].

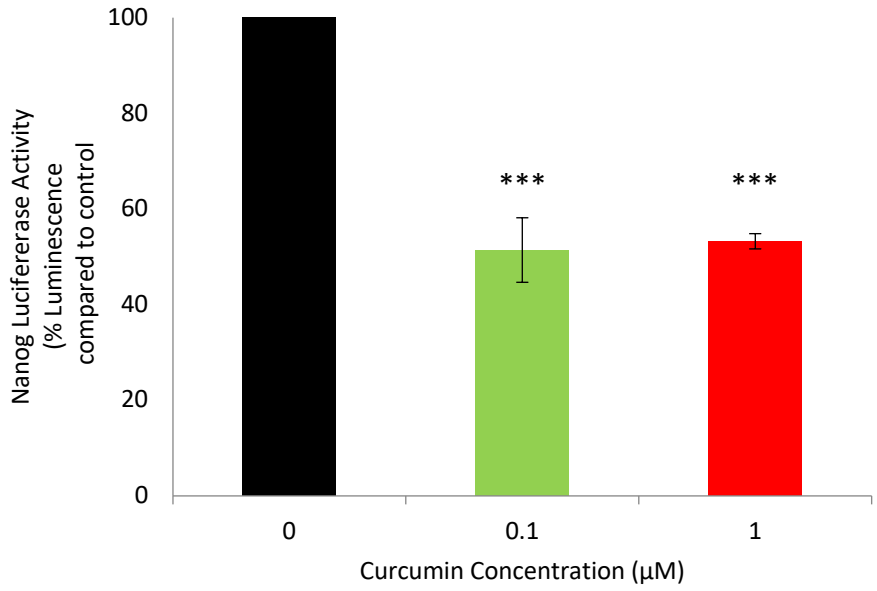


Figure 4-1. Effect of curcumin on Nanog reporter activity in Caco-2 cells. Cells were passaged 1:3 24 hours prior to plating with lipofectamine, plus reagent and inducible Nanog transcription factor responsive firefly and renilla constructs (40:1). Media was changed following 24 hours to avoid toxicity and cells were incubated for a further 24 hours with 0.1 and 1.0 µM curcumin. Values represent mean±SEM of 6 independent experiments (***P<0.001 as determined by student's T Test).

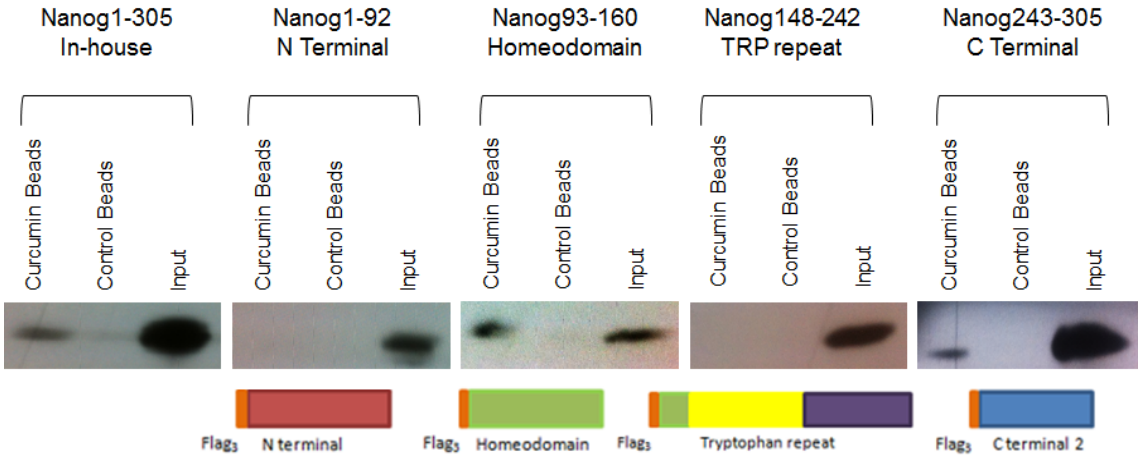


Figure 4-2. Binding of curcumin to Nanog using affinity pull down assay. Curcumin beads pull Nanog form Caco-2 cell lysate and also directly bind the commercially available recombinant Nanog protein (data not shown). Nanog protein1-305 and truncated Nanog proteins were purified in house. Nanog constructs were cloned (DNA constructs generated using pETM6T1Flag vector), expressed in E.Coli and purified using an AKTAPrime system. All constructs were tagged with Flag3 for detection by western blot. Curcumin beads bound to Nanog1-305 and Nanog93-160 purified in house. Abbreviations: TRP: Tryptophan.

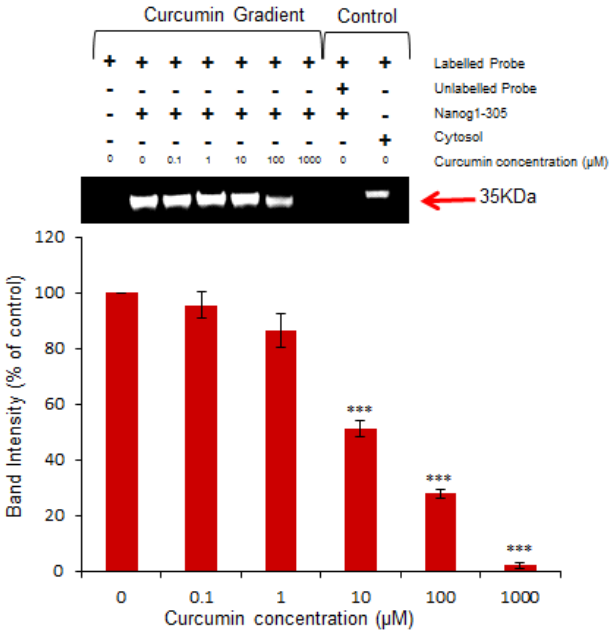


Figure 4-3. Curcumin inhibits Nanog-DNA binding in a dose dependent manner. Electrophoretic mobility shift assays were conducted using fluorescently labelled DNA (containing homeodomin DNA binding motif TAATGG), curcumin and recombinant Nanog1-305 protein (commercially available). (Mean ±SEM triplicate experiments. *p<0.05, **p<0.01***, p<0.001).

4.3 Considering the effect of curcumin on gene expression of Nanog and downstream targets (2D)

To further assess the impact of curcumin on transcriptional activity of Nanog, Nanog gene expression was also assessed. Following treatment of Caco-2 cells with a range of curcumin doses (0-10 μM) no change in Nanog gene expression was detected (see figure 4-4). The effect of curcumin on gene expression of downstream targets of Nanog were also evaluated. These include BMI1 and FAK [202-204]. Others have been reported but these are not expressed in Caco-2 cells e.g. ERSBB or do not directly interact with Nanog [205]. A small significant increase in BMI1 gene expression was detected following treatment at 0.1, 1 and 5 μM curcumin. Though the increase is small and it is statistically significant, the fold change is <0.5 in gene expression [206]. A second downstream target FAK was also considered. Following 0.1 and 1 μM curcumin treatment FAK gene expression increased. Again though this was a statistically significant increase in gene expression, it was less than a 0.5 fold increase (see figure 4-5). It is unclear if a small significant fold change is of importance [206].

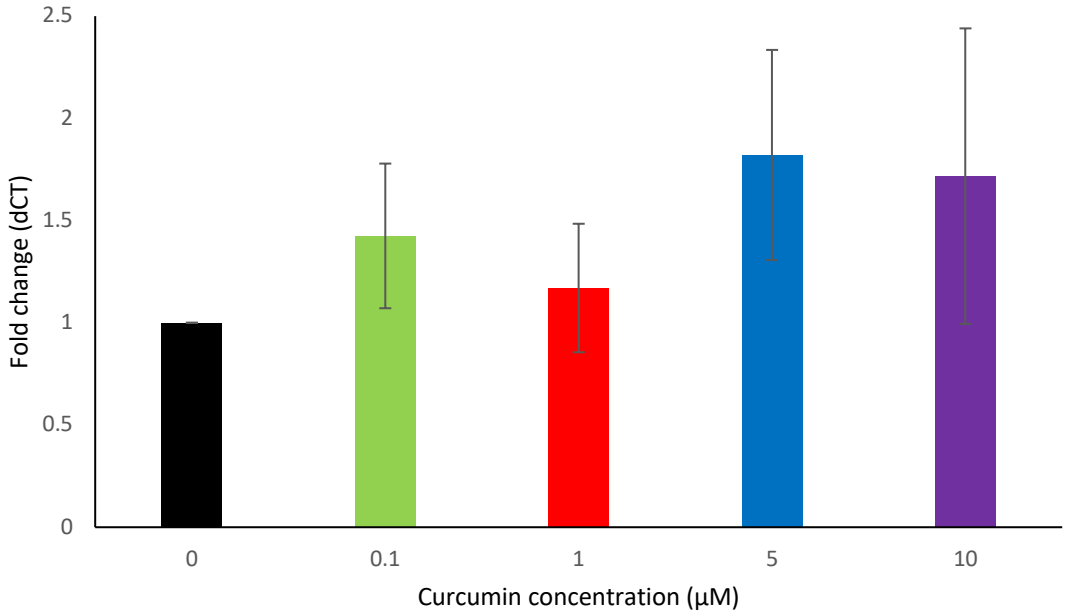


Figure 4-4. Effect of curcumin on Nanog gene expression in Caco-2 cells. Caco-2 cells were treated with a range of curcumin concentrations (0-10 μM) for 24 hours. Following treatment, cells were harvested and analysed for gene expression by qRT-PCR. GAPDH and actin housekeeping genes were used to normalise the data. Values represent fold change compared to vehicle control. Values represent mean±SEM of 3 independent experiments.

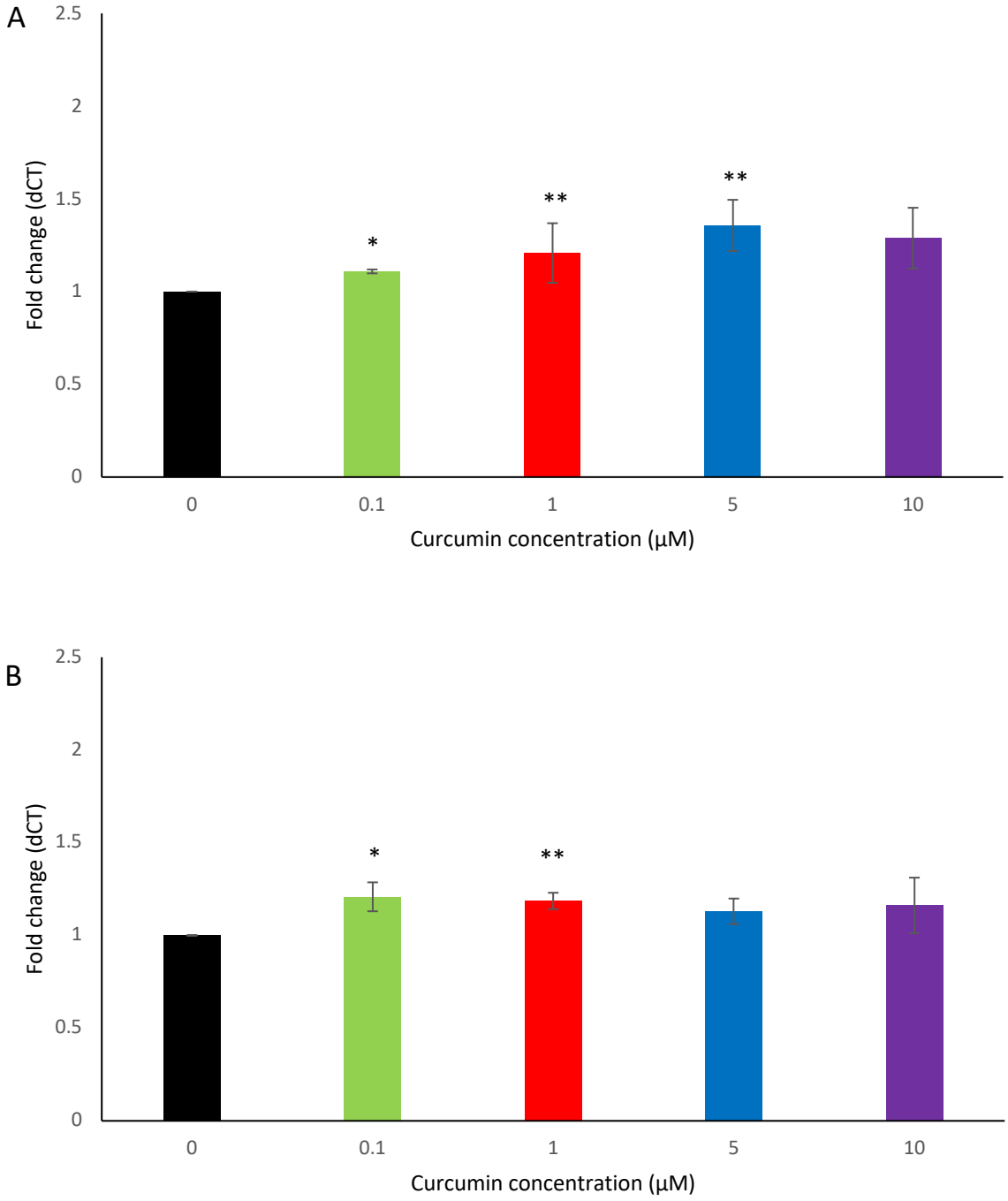


Figure 4-5. Effect of curcumin on BMI1 and FAK gene expression in caco-2 cells. Caco-2 cells were plated, allowed to adhere and treated for 24 hours with curcumin (0-10 µM). Subsequently, cells were harvested and analysed for gene expression by qRT-PCR as previously described. (A) BMI gene expression (B) FAK gene expression. GAPDH and actin housekeeping genes were used to normalise the data. Values represent fold change compared to control (mean±SEM of 3 independent experiments (*P< 0.05, **P<0.01 as determined by student's T Test).

Taken together it may appear that curcumin is not affecting gene regulation of Nanog or its downstream targets. We may fail to see an effect, as curcumin may affect Nanog post-translationally [207, 208]. Published data indicates that phosphorylation at specific sites in the Nanog protein sequence Ser/Thr-Pro motifs, are involved in maintaining the stability of Nanog protein in human embryonic cells [208].

An additional possibility is that the effect of curcumin on CSCs is being masked by the composition of cells being analysed in that there is a mixture of CSCs and non-CSC populations which are not being considered separately. Moreover, if curcumin is able to change the plasticity of CSCs between the CSC and non-CSC phenotype, any changes in the stem cell population will be then result in the overall gene expression to be relatively unchanged. This is a difficult hypothesis to test, as though cells can be sorted into CSC and non-CSC population, as Nanog is an intracellular protein, cells would need to be fixed and permeabilised, prior to sorting. Sorting can feasibly be carried out with PCR on the resulting cells [209-211]. However it is difficult to obtain high quality RNA in sufficient yields to allow PCR to be carried out. There have been methods which exploit the use of high salt buffers during the sorting process which have been shown to help with extraction [211]. As the changes which were likely to be detected were not of a high magnitude, cells were not sorted on Nanog but ALDH activity where *in vivo* data suggesting efficacy of curcumin against this sub-population of CSCs has been observed (as shown in chapter 1) [187].

4.4 Considering the effect of curcumin on protein expression of Nanog and downstream targets (2D)

Nanog protein significantly decreased following curcumin treatment at 0.1, 1 and 10 μM treatment (see figure 4-6). This was also mirrored with a significant decrease in phosphoNanog at the same concentrations (see figure 4-6). However no change was detected in Nanog:PhosphoNanog ratio (see figure 4-7). It may be if cells are sorted into CSC compartment and non-CSC compartment (defined by $ALDH^{high}$ vs $ALDH^{low}$ activity respectively) a biologically relevant and significant change could be detected in Nanog:PhosphoNanog ratio. Moreover, if curcumin is changing the phenotype of the cells from CSC to non-CSC then this change may not be convincingly identified during experimental replicates. Additionally it may be that post translational changes do not play a significant role in the mechanism of action of curcumin. It is worth noting, curcumin is able to effect a multitude of pathways and regulate many different proteins, therefore not all changes are likely to be the primary effect.

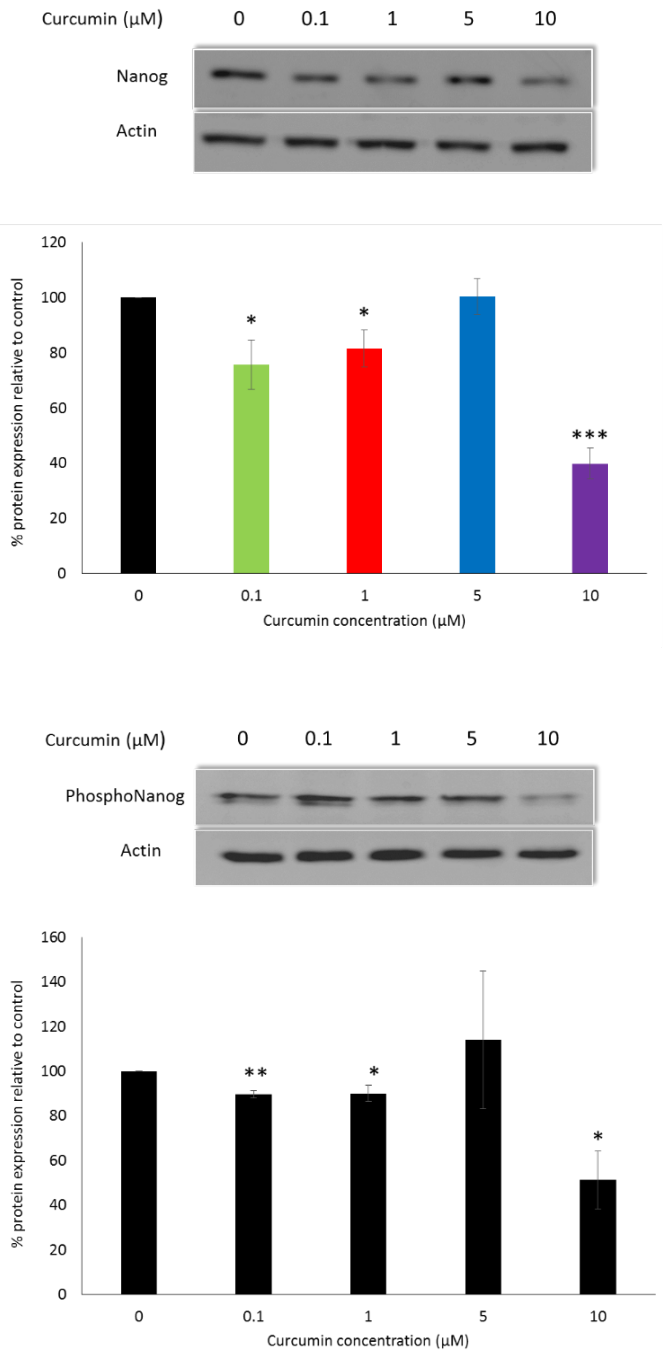


Figure 4-6. Effect of curcumin on Nanog and PhosphoNanog protein expression in Caco-2 cells. Caco-2 cells were exposed to curcumin (0-10 µM) for 24 hours. Subsequently, cells were harvested and Nanog protein or phosphoNanog protein expression was assessed by western blot. Actin was used as a loading control for normalisation. Three separate biological replicates were performed on different occasions. Values are expressed as a % relative to control and represent mean±SEM triplicate experiments (*P< 0.05 **P<0.01 ***P<0.001 as determined by student's T Test).

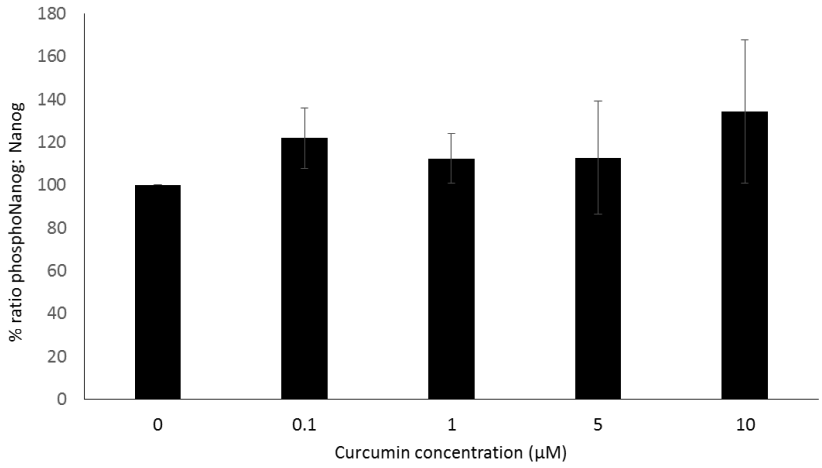


Figure 4-7. Effect of curcumin on Nanog:PhosphoNanog protein ratio in Caco-2 cells. Caco-2 cells were exposed to curcumin (0-10 μM) for 24 hours. Subsequently, cells were harvested and Nanog protein or phosphoNanog protein expression was assessed by western blot. Nanog:PhosphoNanog ratio was calculated. Actin was used as a loading control for normalisation. Three separate biological replicates were performed on different occasions. Values are expressed as a % relative to control and represent mean±SEM triplicate experiments.

The effect of curcumin on Oct4, another CSC regulating protein was also considered. No change in protein levels was detected (figure 4-8). It is possible that curcumin has no activity against this CSC protein or is unable to change its regulation. This indicates a specificity for the Nanog pathway.

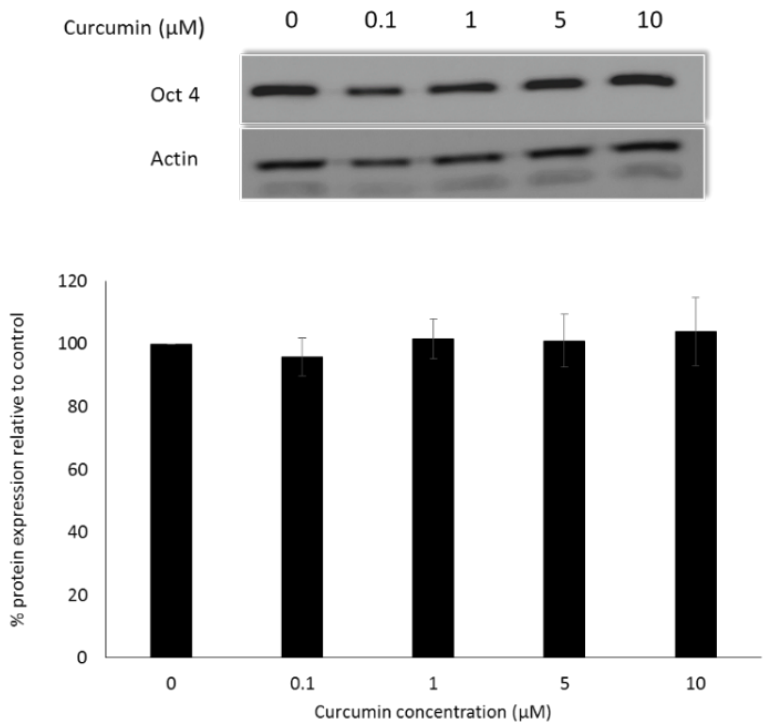


Figure 4-8. Effect of curcumin on Oct4 protein expression in Caco-2 cells. Caco-2 cells were exposed to curcumin (0-10 μM) for 24 hours. Subsequently, cells were harvested and Oct4 protein expression was assessed by western blot. Actin was used as a loading control for normalisation. Three separate biological replicates were performed on different occasions. Values are expressed as a % relative to control and represent mean±SEM triplicate experiments.

Subsequently, the effect of curcumin on the protein expression of Nanog's downstream targets was assessed following 24 hours treatment (figure 4-9). Here, there was a significant decrease in BMI protein expression at 0.1, 1 and 5 μM treatment of 20-30%. In addition, there was upto 50% decrease in FAK protein expression at 0.1, 1 and 5 μM treatment. This suggests, targets which are downstream of Nanog are also affected following curcumin treatment in addition to the Nanog protein. Although gene expression remains unchanged. This may be as a result of curcumin affecting the plasticity of CSCs i.e. Nanog containing cells are pushed into differentiation resulting in decreased Nanog protein and downstream targets. This was detected with 24 hour treatments. However no gene changes were detected. It is possible this is due to post translational modification of proteins. In addition, it was worth noting the effect on protein expression was not a linear dose response effect that is often seen with targeted or cytotoxic chemotherapies. A greater effect is seen at lower concentrations compared to the highest concentration used of 10 μM.

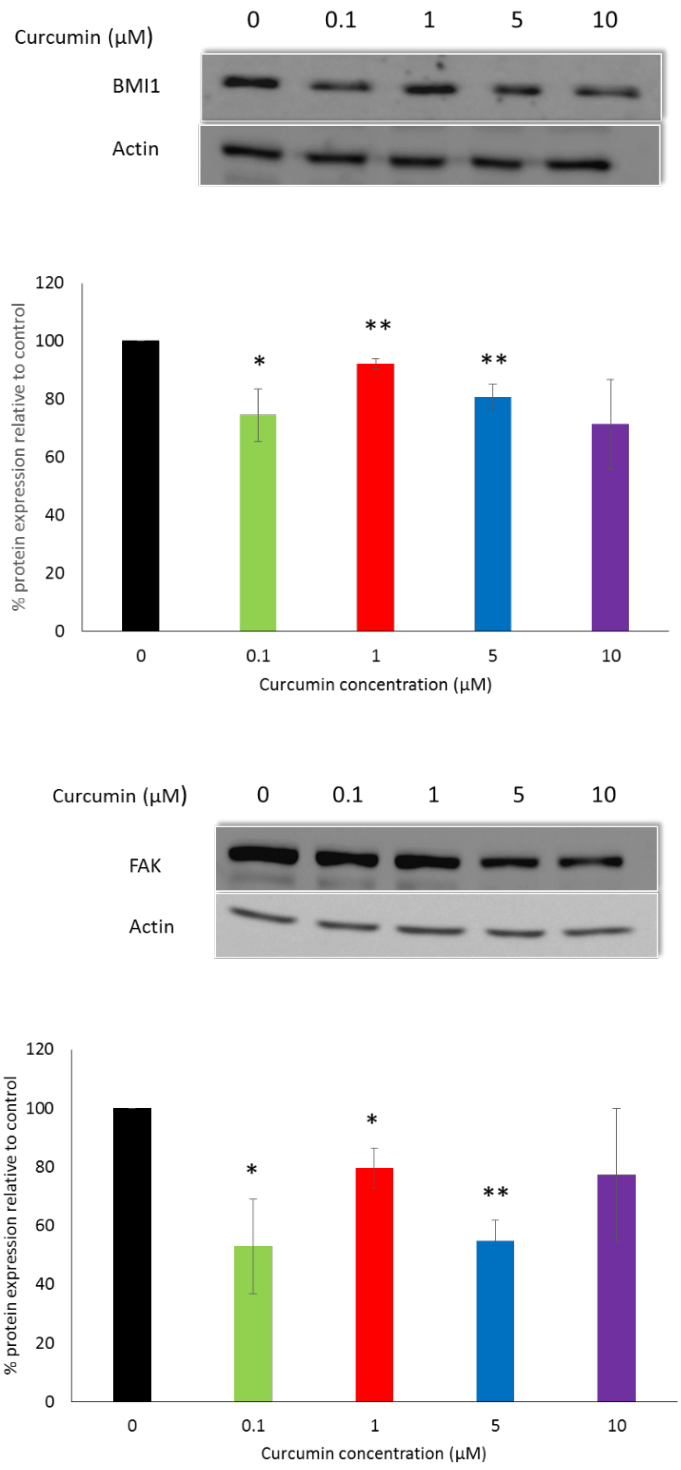


Figure 4-9. Effect of curcumin on BMI1 and FAK protein expression in Caco-2 cells. Caco-2 cells were exposed to curcumin (0-10 μM) for 24 hours. Subsequently, cells were harvested and BMI1 and FAK protein expression was assessed by western blot. Actin was used as a loading control for normalisation. Three separate biological replicates were performed on different occasions. Values are expressed as a % relative to control and represent mean±SEM triplicate experiments (*P< 0.05 **P<0.01 ***P<0.001 as determined by student's T Test).

4.5 Investigating the effect of curcumin on Nanog$^+$ expression and Nanog cell proliferation (Nanog$^+$Ki67$^+$) in Caco-2 cells (2D)

A key concern identified with experiments examining the effect of Nanog within a CSC and non-CSC population i.e. a mixed population of cells, is that it is difficult to elucidate with confidence the effects on this population only. To overcome this, a flow cytometry based assay was used, allowing proliferation in only the Nanog$^+$ subpopulation to be determined.

Following curcumin treatment, a 20-30% reduction in Nanog$^+$ expression was detected following 24 hour incubation (figure 4-10). This was significant at 0.1, 1 and 5 μM and correlated with protein expression analysed by western blot. In addition, when the proliferating proportion of Nanog$^+$ cells (Nanog$^+$Ki67$^+$) was assessed, there was a 30-50% significant reduction at 0.1, 1 and 5 μM, suggesting not only that the CSC total compartment is affected, but there is a reduction in the proliferative capacity of the CSC population (figure 4-11). This is helpful, as not only is curcumin possibly changing the phenotype of CSCs into non-CSCs it is also inhibiting the division of CSCs, further reducing this population.

In the non-CSC proliferating population (Nanog$^-$Ki67$^+$) no change in response to curcumin treatment was observed (figure 4-12). It hypothesised curcumin may induce a CSC to non-CSC switch, thereby increasing the Nanog$^-$Ki67$^+$ population, however this was not detected. This may be because the proportion of Nanog$^+$ cells in Caco-2 cells was approximately 5-15% over the three independent replicates performed. Similarly to western blot observations, a non-linear dose response was observed.

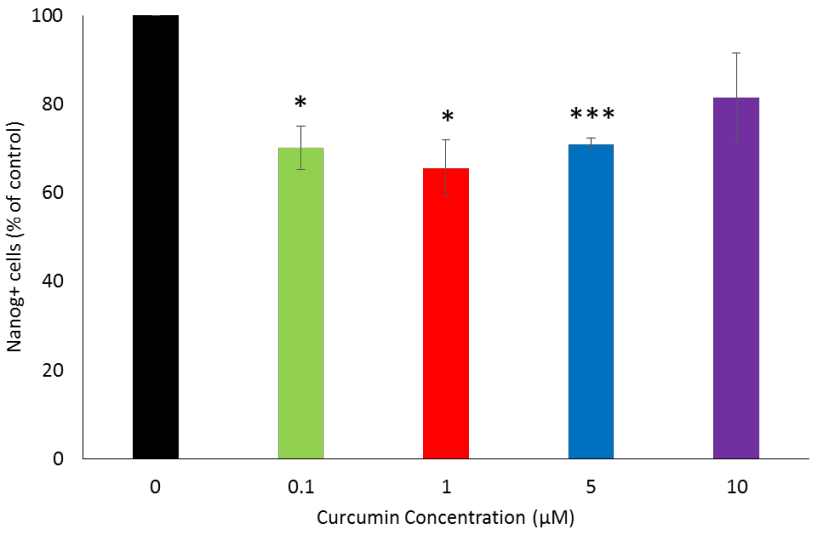


Figure 4-10. Effect of curcumin on Nanog$^+$ cell population in Caco-2 cells. Cells were plated, allowed to adhere and were treated with curcumin (0-10 µM) for a period of 24 hours. Cells were then harvested, fixed and permeabilised and antibodies added. Cells were analysed and the proportion of Nanog$^+$ cells assessed. All values are expressed as a % relative of respective solvent control and mean±SEM of triplicate experiments (*p<0.05, *p<0.001, ***p<0.001 as determined by students T-Test).

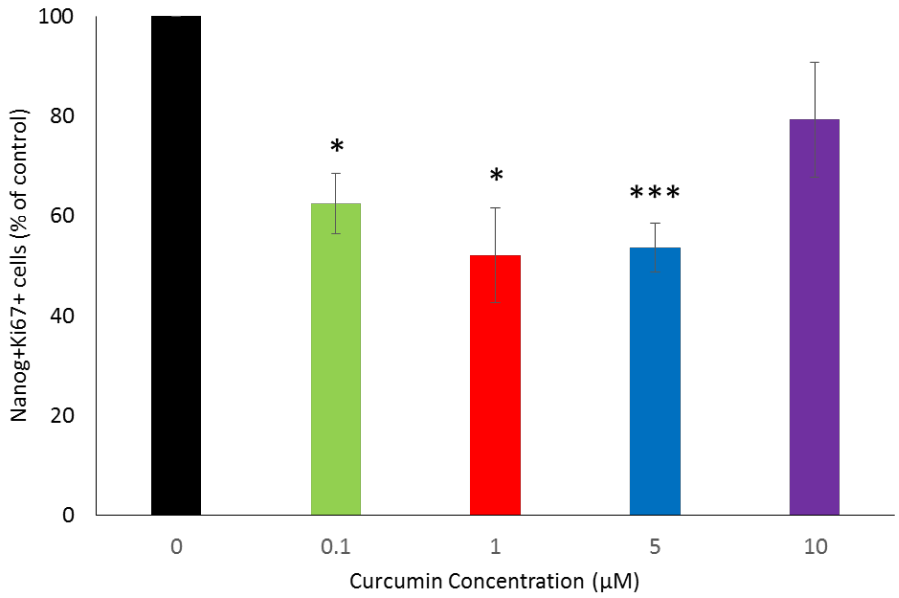


Figure 4-11. Effect of curcumin on Nanog$^+$Ki67$^+$ cell population in Caco-2 cells. Cells were plated as previously described and treated with curcumin (0-10 µM). All values are expressed as a % relative of respective solvent control and mean±SEM of triplicate experiments (*p<0.05, *p<0.001, ***p<0.001 as determined by students T-Test).

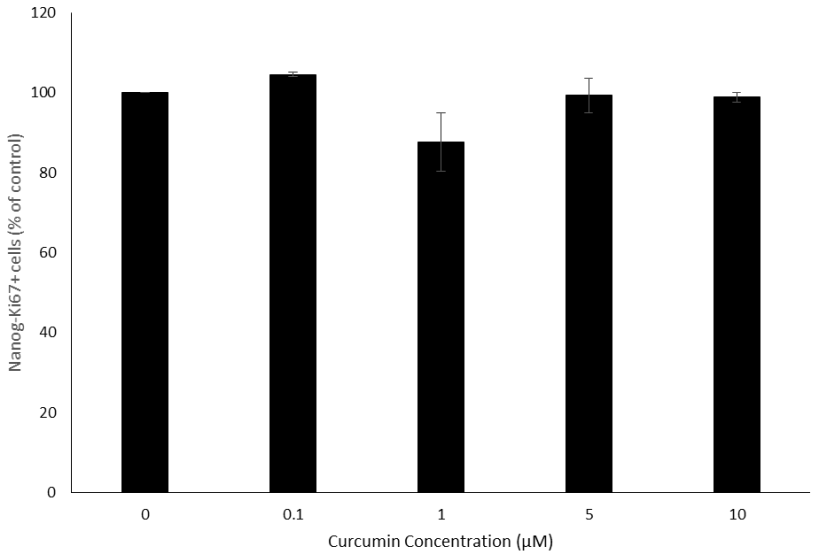


Figure 4-12. Effect of curcumin on Nanog^{-}Ki67^{+} cell population in Caco-2 cells. Cells were treated as previously described. All values are expressed as a % relative of respective solvent control and mean±SEM of triplicate experiments.

4.6 Investigating the effect of curcumin on gene expression of Nanog and downstream targets in CSC and non-CSC compartment (defined by ALDH^{high} vs ALDH^{low} activity respectively) (2D)

An alternative method for characterising the CSC population is to cell sort. Whilst Nanog would be the marker of preference for sorting, RNA extraction can be difficult from fixed and permeabilised cells as the yield, purity and integrity can be affected markedly [212]. Furthermore, Nanog is expressed at low levels in caco-2 cells, so in order to obtain enough material for subsequent western blotting, the amount of start-off material required would be unfeasible. To this end, as we have previously shown that ALDH represents a robust CSC marker, and has the added advantage that it can be identified in live cells rather than those that are fixed and permeabilised, I chose to sort populations based on ALDH expression. Despite this, it remained difficult to obtain sufficient quantities and quality of RNA.

Firstly, gene expression was reassessed for Nanog and downstream markers in ALDH^{high} and ALDH^{low} sorted cells. Again no difference was detected following curcumin treatment with 0.1 μM (figure 4-13). This dose of curcumin was chosen because efficacy

was seen in a wide range of previous assays at this dose, as well as in transcriptional activity and biophysical assays.

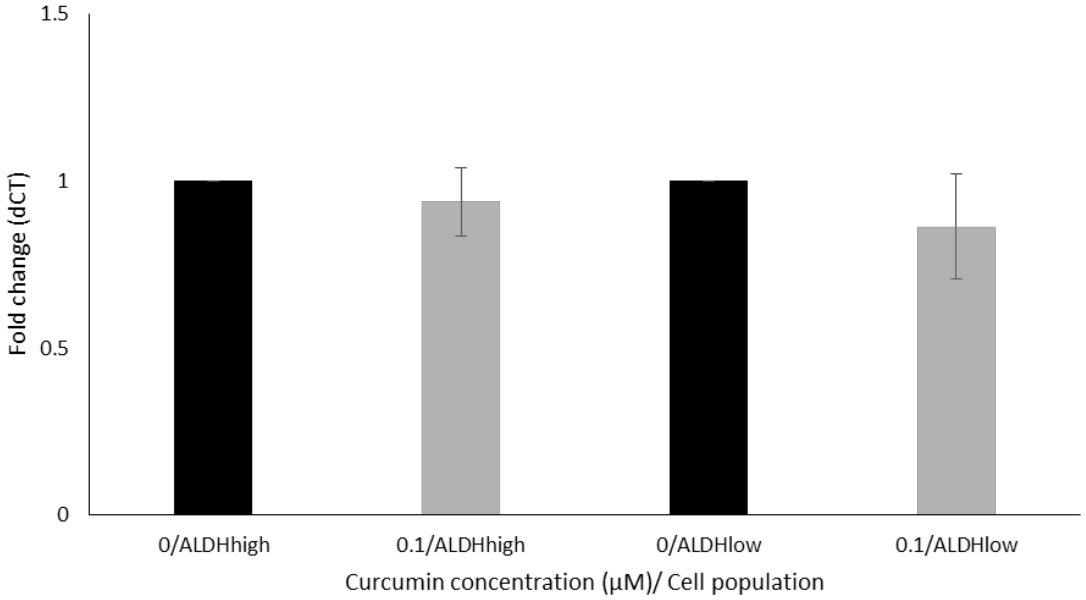


Figure 4-13. Effect of curcumin on Nanog gene expression in ALDH^{high} and ALDH^{low} Caco-2 cells. Caco-2 cells were treated with 0.1 µM curcumin for 24 hours and harvested. They were stained with aldefluor and cell sorted using a FACS Aria II into ALDH^{high} (CSC compartment) and ALDH^{low} (non-CSC compartment) using appropriate gates and controls. Cells were analysed for gene expression by qRT-PCR. GAPDH and actin housekeeping genes were used to normalise the data. Values represent fold change compared to vehicle control 0 µM. Values represent mean±SEM of 3 independent experiments.

Subsequently, gene expression in downstream targets were also assessed following treatment and cell sorting as described above. In contrast to non-sorted cells, gene expression of BMI1 was significantly downregulated by curcumin, although the fold change was <0.5 (figure 4-14). Gene expression of FAK was also significantly lower compared to control in the sorted population. Again fold change <0.5 (figure 4-15). The biological relevance of a small fold change is unclear [206]. It has been suggested a fold change of 2-3 is more likely to be of importance than a smaller fold change, therefore the importance of this significant result is unclear.

It would be advantageous to also investigate a range of concentrations of curcumin using this methodology, however, a better system would be a system which considers

Nanog specifically. One option would be to look at a Nanog over-expressing colorectal cell line or a Nanog knock-down colorectal cancer cell line.

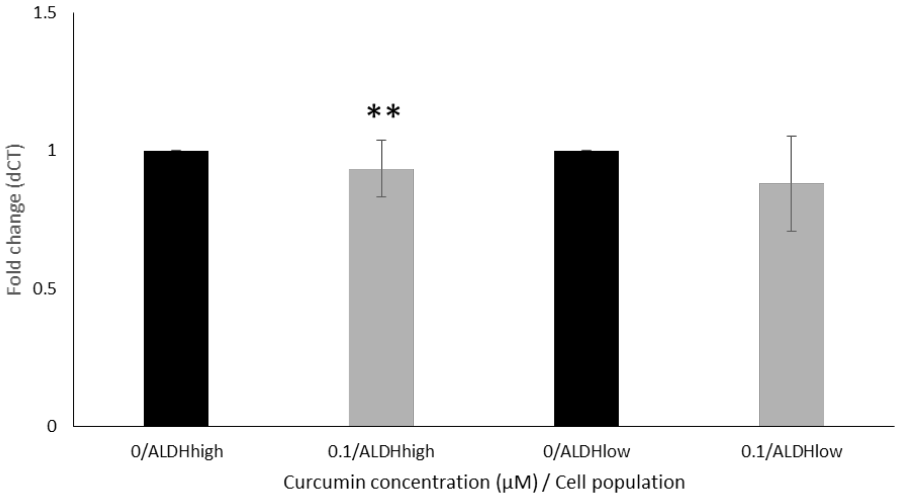


Figure 4-14. Effect of curcumin on BMI1 gene expression in ALDH^{high} and ALDH^{low} Caco-2 cells. Caco-2 cells were treated with 0.1 µM curcumin for 24 hours and harvested. They were stained with aldefluor and cell sorted using a FACS Aria II into ALDH^{high} (CSC compartment) and ALDH^{low} (non-CSC compartment) using appropriate gates and controls. Cells were analysed for gene expression by qRT-PCR. GAPDH and actin housekeeping genes were used to normalise the data. Values represent fold change compared to vehicle control 0 µM. Values represent mean±SEM of 3 independent experiments (**p<0.001 as determined by students T-Test).

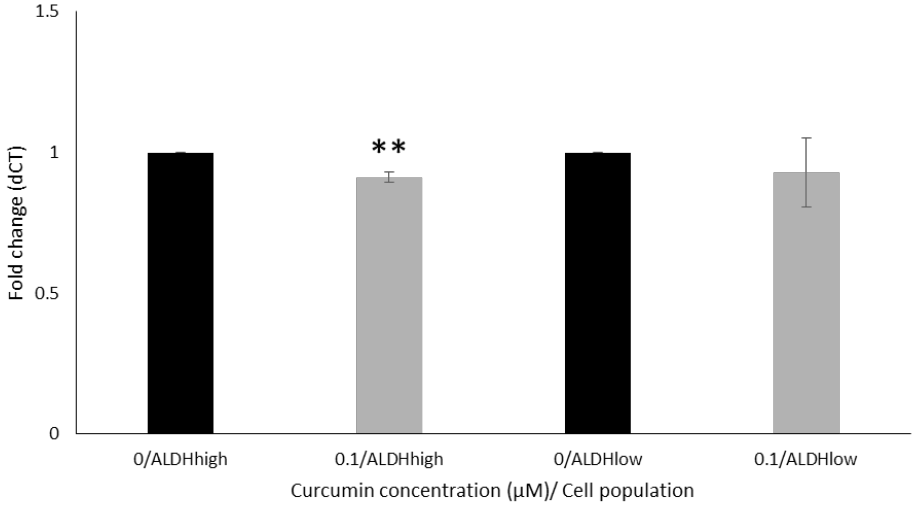


Figure 4-15. Effect of curcumin on FAK gene expression in ALDH^{high} and ALDH^{low} Caco-2 cells. Caco-2 cells were treated, sorted and analysed for gene expression by qRT-PCR. GAPDH and actin housekeeping genes were used to normalise the data. Values represent fold change compared to vehicle control. Values represent mean±SEM of 3 independent experiments (**p<0.001 as determined by students T-Test).

4.7 Investigating the effect of curcumin on protein expression of Nanog and downstream targets in CSC compartment and non-CSC compartment (defined by ALDH^{high} vs ALDH^{low} activity respectively) (2D)

Though no change in Nanog gene expression was identified following curcumin treatment in caco-2 cells, a significant decrease in Nanog protein expression was identified in the CSC compartment only (~50% reduction) (figure 4-16). This suggests that the effects observed in non-sorted cells is due to an effect confined to the CSC fraction. The non-significant reduction in the non-CSC population may be due to the fact that the cells were not sorted by Nanog but by ALDH, meaning that it is possible some Nanog^{+} cells remain in the non-CSC compartment. In addition it was difficult to carry these experiments out due to problems with obtaining enough material As a result western blot quality and RNA quality was not optimum and results are difficult to interpret.

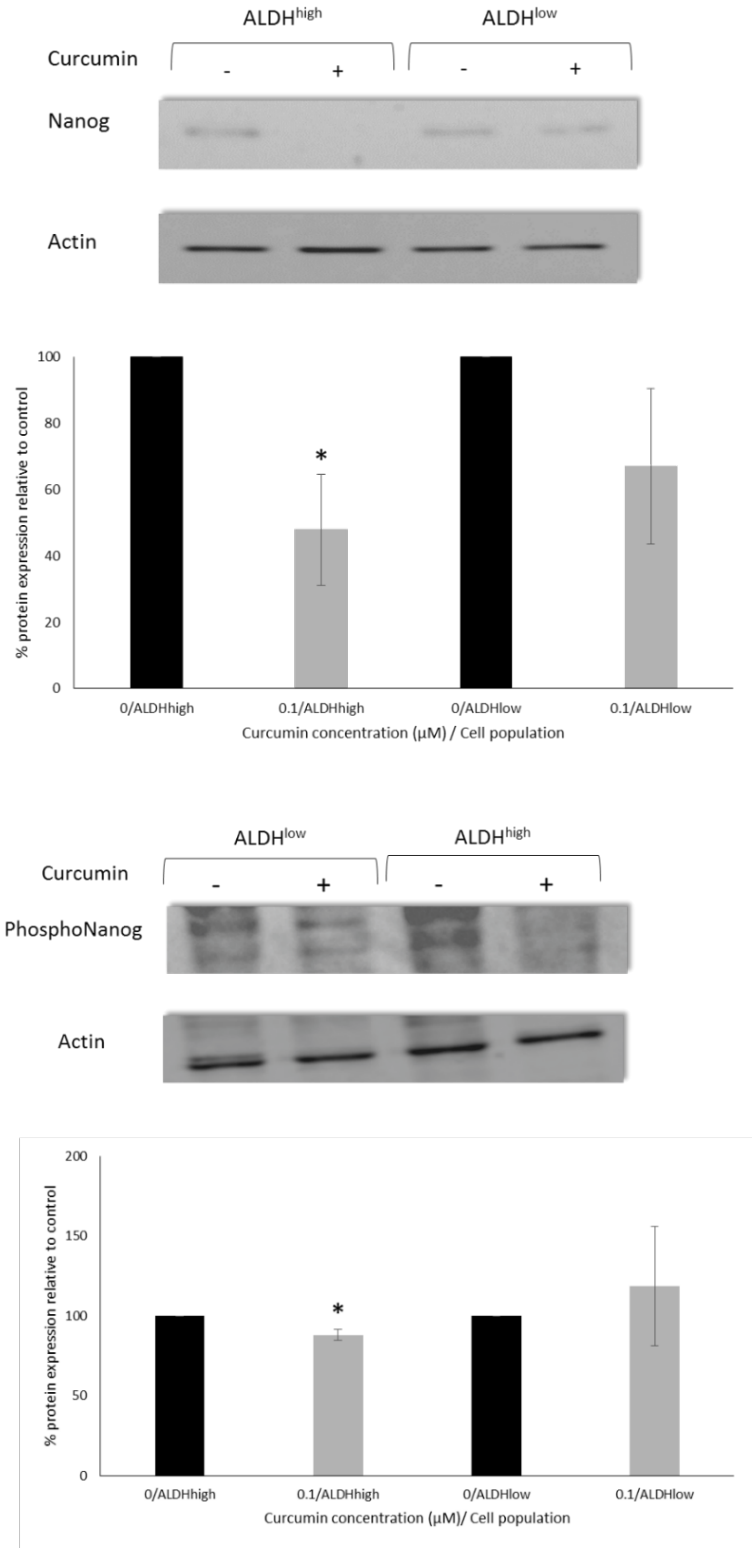


Figure 4-16. Effect of curcumin on Nanog and PhosphoNanog protein expression in ALDH^{high} and ALDH^{low} Caco-2 cells. Caco-2 cells were treated, sorted and analysed for Nanog protein expression by western blot. Actin was used as a loading control for normalisation. Values represent mean±SEM of 3 independent experiments (*p<0.005 as determined by students T-Test).

Phosphorylation status of Nanog – serine 71 was also considered (figure 4-17). A decrease in phosphorylation in the CSC compartment was observed (<10%), but this was not the case for the non-CSC compartment, where there was no change. When the ratio of PhosphoNanog to Nanog was calculated, a small significant reduction (<10%) was observed, suggesting curcumin is able to destabilise Nanog protein. However, notably bands were difficult to detect due to difficulties with obtaining sufficient quantities of material.

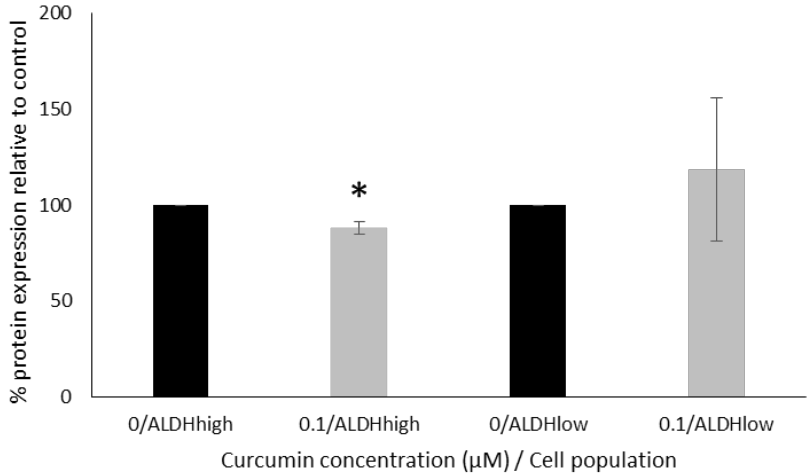


Figure 4-17. Effect of curcumin on PhosphoNanog:Nanog ratio in ALDH^{high} and ALDH^{low} Caco-2 cells. Caco-2 cells were treated, sorted and analysed for Nanog and PhosphoNanog protein expression by western blot as previously described. A ratio between the two proteins was calculated. Actin was used as a loading control for normalisation. Values represent mean±SEM of 3 independent experiments (*p<0.05 as determined by students T-Test).

In addition to consider the specificity of this interaction, another CSC regulating protein Oct4 was considered (figure 4-18). No effect was seen following curcumin treatment in the CSC or non-CSC compartment. This is in keeping with the effect seen when non-sorted caco-2 cells were treated with curcumin.

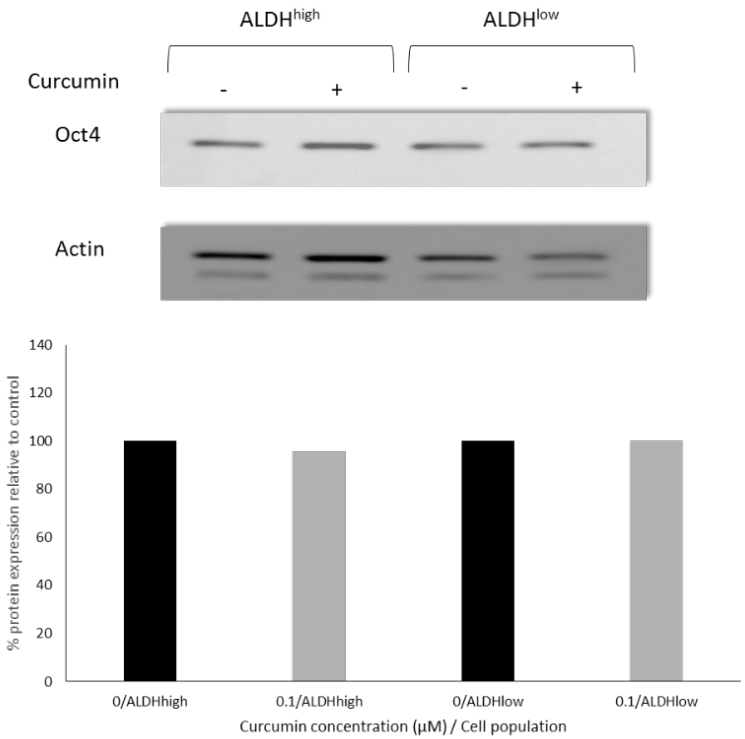


Figure 4-18. Effect of curcumin on Oct4 expression in ALDH^{high} and ALDH^{low} Caco-2 cells. Caco-2 cells were treated, sorted and analysed for Oct4 protein expression by western blot. Actin was used as a loading control for normalisation. Values represent mean±SEM of 3 independent experiments.

Subsequently, the effect of curcumin on downstream targets of Nanog in a CSC and non-CSC population was assessed (figure 4-19). A significant decrease in BMI1 and FAK protein expression was identified only in the CSC compartment, to a greater magnitude than that seen in the non-sorted cells, suggesting non-CSC cells were masking some of the effect seen.

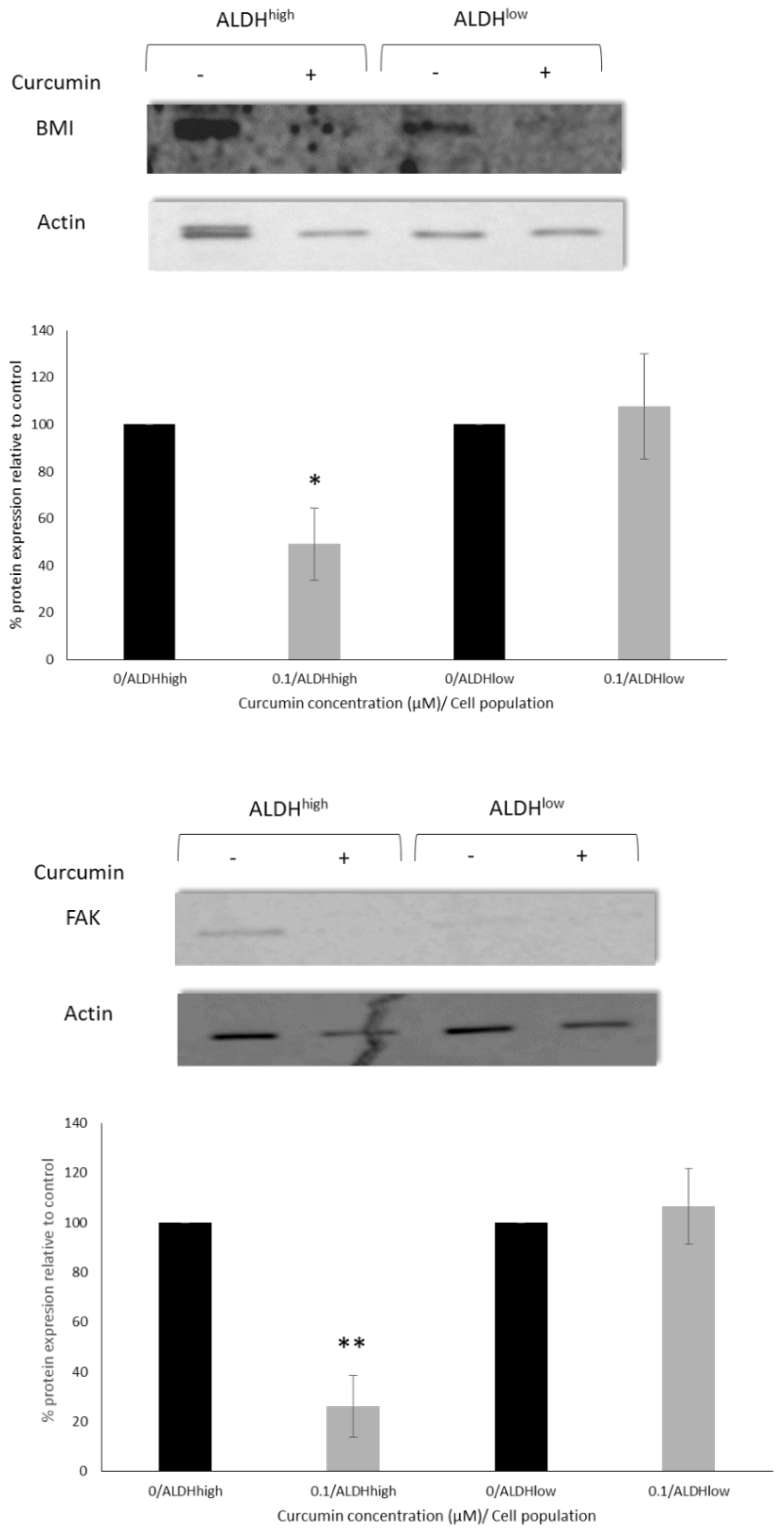


Figure 4-19. Effect of curcumin on BMI1 and FAK protein expression in ALDH^{high} and ALDH^{low} Caco-2 cells. Caco-2 cells were treated, sorted and analysed for downstream targets of Nanog protein by western blot. Actin was used as a loading control for normalisation. Values represent mean±SEM of 3 independent experiments (*p<0.05, **p<0.01 as determined by students T-Test).

4.8 Conclusion

The results in this chapter indicate curcumin is able to modify the CSC and non-CSC population in cancer cell lines. As curcumin is known to modulate a number anti-cancer pathways, this is in keeping with published literature [213-215]. Interestingly, curcumin is able to decrease the transcriptional activity of Nanog within 24 hours of treatment. It is unclear if the reduction in activity seen is as a result of a decrease in the total population of cells as a result of curcumin treatment such as via apoptosis, though at very low concentrations, apoptosis is unlikely [216, 217], or as a result of selective activity against the CSC population at low concentrations e.g. inducing CSC plasticity, so CSCs are pushed into a non-CSC population. Other considerations include post translational effects on Nanog protein in CSCs resulting in a decreased Nanog population [202, 218].

As a mixed population of cells is used, it is difficult to decipher the effect of curcumin on the CSC vs non-CSC population. Methods to assess the CSC population in this assay were considered. These included sorting cells into a Nanog vs non-Nanog population, however this would require intracellular staining, rendering the cells dead. In addition, sorting cells into $ALDH^{high}$ and $ALDH^{low}$ populations and using the $ALDH^{high}$ population was considered. However due to the nature of the assay i.e. cells are required to adhere for 24 hours with lipofectamine, need acclimatisation in media for a further 24 hours and treatment for 24 hours, it is likely a CSC population which has been cell sorted will begin to differentiate during this time as cell lines have a high replicating capacity, without curcumin treatment. In addition, the auto-fluorescence of aldefluor sorted cells, coupled with the effect of curcumin on the assessment of the cignal reporter assay would make changes as a result of curcumin in this population very difficult to assess.

The effect of curcumin on the gene expression of Nanog and downstream targets was assessed in unsorted caco2 cells, and $ALDH^{high}$ and $ALDH^{low}$ populations. Here very little change was detected. In some systems, mRNA expression does not always correlate with protein expression [219]. Alternative mechanisms such as post translational changes and protein degradation may account for the lack of correlation. Therefore, it is difficult to make inferences on gene expression as a result of minimal change following curcumin treatment. In addition there was limited material to carry assays out with which could affect the results.

The effect of curcumin on protein expression of Nanog was assessed in unsorted and sorted cells. Here a statistically significant reduction was detected in Nanog protein expression following curcumin treatment in a U-shaped dose response in non-sorted cells (20-30%). No change was detected in Nanog:Phosphonanog expression. A greater significant reduction in Nanog was detected in $ALDH^{high}$ population than non-sorted cells (approximately 50%). This may be due to more selective targeting of cells [220]. In addition there was limited material to carry assays out with which could affect the results. Non- significant changes were detected in the $ALDH^{low}$ population. This was supported by the flow cytometry data, assessing effects on $Nanog^{+}$, $Nanog^{+}Ki67^{+}$ and $Nanog^{-}Ki67^{+}$ populations.

The data presented suggests Nanog may be targeted by curcumin whilst Oct4 is not. However, this is a 2D cell culture system, therefore has not employed sphere culture/ 3D culture. Additionally, though the CSC and non-CSC population have been considered, a Nanog enriched population, or a Nanog knockdown model was not used. These two possibilities will be considered in the next chapter of work, which will allow validation of the markers detected here.

Lastly, the safety of curcumin and its ability to be taken for a long periods of time appears to be tolerable, whether or not it affects the development and fate of normal cancer stem cells has not been studied as extensively as cancer stem cells. Possible explanations for why curcumin is able to target CSCs but not normal healthy stem cells may be due to a differential uptake of curcumin between the two populations [220]. An interesting experiment involving assessment of the fluorescence spectra of curcumin-loaded cells in two normal cell lines and two malignant cell lines was assessed. Uptake was much higher in the malignant cell lines suggesting curcumin is able to accumulate more readily. In addition, curcumin has been observed to affect the microenvironment as well as cells themselves. It appears changes in the microenvironment seem to favour the development of normal stem cells rather than CSCs, e.g. curcumin decreases the release of pro-inflammatory cytokines [220]. Lastly, there has been suggestion that curcumin may not be directly toxic to CSCs and instead of inducing apoptosis or cell death mechanisms, curcumin induces differentiation. This has been suggested as a method of eliminating the CSC population in the literature [220].

The data in this chapter suggests, curcumin is able to target CRC CSCs. This work was carried out in 2D short term cultures and did not specifically consider cells which express

Nanog. In the next chapter, longer term 3D cultures using a Nanog enriched population of cells will be considered.

5.0 Chapter 5 (Results and Discussion) – Effect of curcumin on modulation of cancer stem-like cells using Nanog overexpressing cells

5.1 Introduction

To investigate the effect of curcumin specifically on cells with high Nanog expression, a cell line was donated to the project which had been engineered to over-express Nanog ($HCT116^{GFP/Nanog}$) with a matched control $HCT116^{GFP}$. Traditionally, to test drug efficacy, a knock-out cell line is generated [221]. However, due to the cellular properties of Nanog, it is technically and practically unfeasible to knock down both Nanog P1 and P8 in the same population of cells and grow long-term sphere cultures. Either can be knocked down independently, using the CRISPR-Cas9 system with recent successes in some prostate cancer cell lines [222]. Due to these difficulties, the over-expression system was used.

Although it has been shown that cellular changes can be seen as early as 24 hours following curcumin treatment in 2D cultures (reporter assay and flow cytometry on caco2 cells), it is unclear how this translates to 3D cultures. A spheroid culture system was used, as this is a surrogate marker for CSCs and an established culture system within the literature. In addition, there is evidence to support primary cancers maintain the expression of stem-like markers in culture suggesting results may be correlated between the two methodologies [223]. Critically, therapeutic prevention, requires the need for long-term treatment, and this was also considered in these experiments.

5.2 Ability of $HCT116^{GFP}$ and $HCT116^{GFP/Nanog}$ cells to form spheres and effect of curcumin (3D)

Prior to testing the effect of curcumin on either cell line, the ability of each cell line to form spheres was assessed. Initially a range different cell numbers were plated (1000-15000 cells per well) and their ability to sphere and reach confluency was assessed weekly over a period of 4 weeks. Subsequently, optimum conditions were established: 1000 cells were plated per well for a period of 2 weeks (this time frame allowed adequate sphere formation without them becoming overly confluent). During this time, it was clear

that HCT116$^{GFP/Nanog}$ cells were able to form spheres more readily compared to HCT116^{GFP} cells (see figure 5-1 and 5-7 showing representative pictures of long-term cultures). To establish the effect of curcumin on sphere generation, 1000 cells were plated into curcumin-containing media at a range of concentrations (0-10 μM). Media was supplemented with curcumin on alternate days for a further 4 treatments. After a period of 24 hours following the final treatment, spheres were harvested and plated onto slides allowing them to be independently counted by an assessor blinded to the treatment regimen. This was carried out in triplicate, with results normalised to control for each cell line respectively. Not only were HCT116$^{GFP/Nanog}$ cells able to sphere more readily than the HCT116^{GFP} cell line, they were significantly more sensitive to curcumin treatment when compared to respective solvent control. A significant difference was observed between treatments for both cell lines from 0.1 μM (20-50% reduction in sphere numbers for HCT116^{GFP} and a 50-80% reduction in sphere number for HCT116$^{GFP/Nanog}$). Although a specific mechanism of action cannot be inferred from these results, this ability to inhibit sphere formation as a surrogate for stemness, further suggests curcumin is able to target the CSC phenotype.

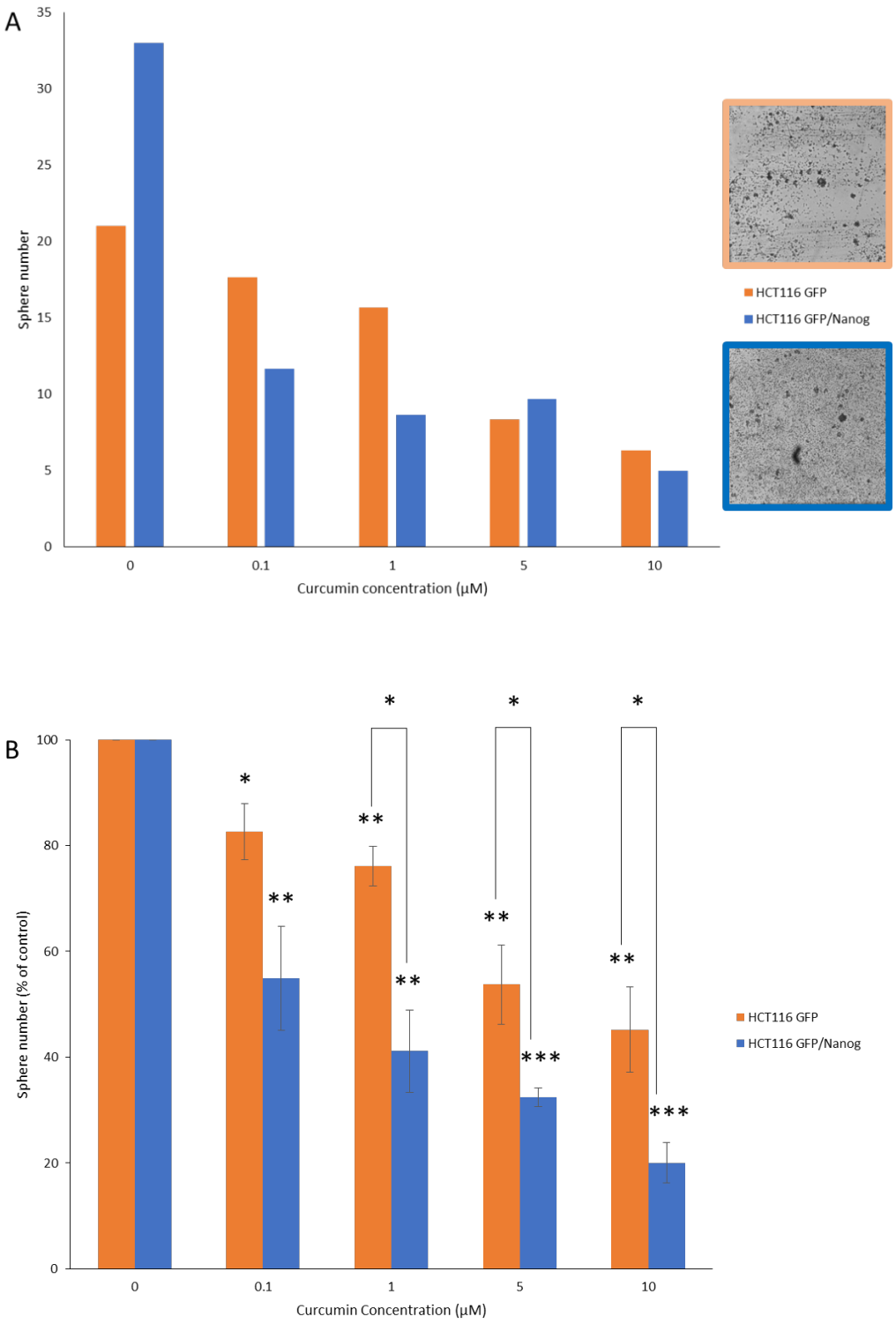


Figure 5-1. Effect of curcumin on sphere formation of HCT116$^{/GFP}$ and HCT116$^{GFP/Nanog}$ cells. Single cells (1000/well) were plated in six well ultra-low attachment plates in curcumin containing stem cell media. Cells were treated with curcumin on alternate days for a further 4 treatments. Following treatment spheres were counted. Data represent a single replicate (A) and triplicate data (B). All values represent mean±SEM (*p<0.05, **p<0.001, ***p<0.001 as determined by students T-Test).

5.3 Determining effect of curcumin on CSCs ($Nanog^+$), CSC proliferation ($Nanog^+Ki67^+$, $Nanog^-Ki67^+$ and $Nanog^-Ki67^-$) and apoptosis ($Nanog^+Caspase^+$ and $Nanog^-Caspase^-$) in $HCT116^{/GFP}$ and $HCT116^{GFP/Nanog}$ cells (3D)

To assess the effect of curcumin on Nanog expression, proliferation and apoptosis in CSC-enriched populations, curcumin-treated spheres were harvested and the single cells stained for Nanog, Ki67 and caspase-3 expression. Examples of plots are shown in figure 5-2.

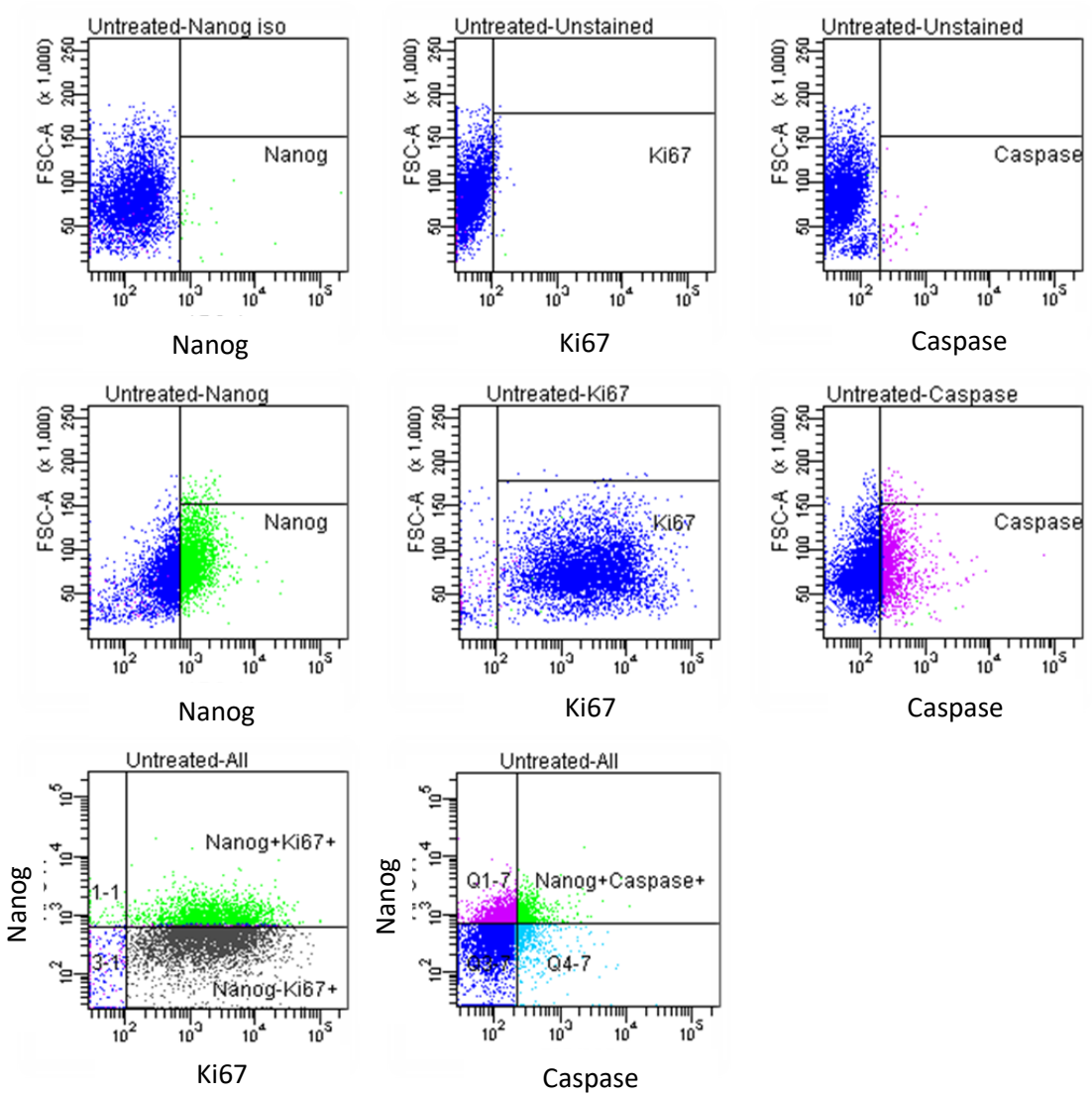


Figure 5-2. Gating strategy used to identify Nanog$^+$, Nanog$^+$Ki67$^+$, Nanog$^-$Ki67$^+$ and Nanog$^+$Caspase$^+$ cells. Single cells were plated in six well ultra-low attachment plates in curcumin containing stem cell media. Cells were treated with curcumin on alternate days for a further 4 treatments. Following treatment, spheres were harvested and single cells created. Subsequently, cells were fixed and permeabilised, antibodies added and analysis carried out. (Top) Unstained population identified (Middle) Isotype used to identify Nanog$^+$ cells, unstained sample for Ki67$^+$ cells and Caspase$^+$ cells identified (Bottom) Nanog$^+$Ki67$^+$, Nanog$^-$Ki67$^+$ and Nanog$^+$Caspase$^+$ cells identified. Experiments were carried out in triplicate, at independent times, using separate passage numbers and spate sphere cultures.

Following curcumin treatment the percentage of Nanog^{+} expressing cells were significantly reduced when compared to solvent control in a dose-dependent manner (15-50% reduction in sphere numbers for HCT116^{GFP} and a 20-75% reduction in sphere number for HCT116$^{GFP/Nanog}$). There was a significant difference between cell lines at 10μM treatment (see figure 5-3). This difference however, was not as marked as that observed in sphere numbers between cell lines. Thus may be due to a differential sensitivity between the cell lines when seeded directly into curcumin-containing media, with fewer HCT116^{GFP} surviving therapy. It is plausible that additional peripheral or alternative pathways are affected with curcumin treatment. The baseline expression of Nanog^{+} cells in the HCT116^{GFP} cell line were 52% (average over 4 replicates) and in the HCT116$^{GFP/Nanog}$ cell line were 71% (average over 4 replicates). Puromycin was used to initially select for cells in a 2D setting. They were harvested and plated in stem cell media and the selection was no longer used. This was to ensure assays were able to detect changes as a result of curcumin treatment.

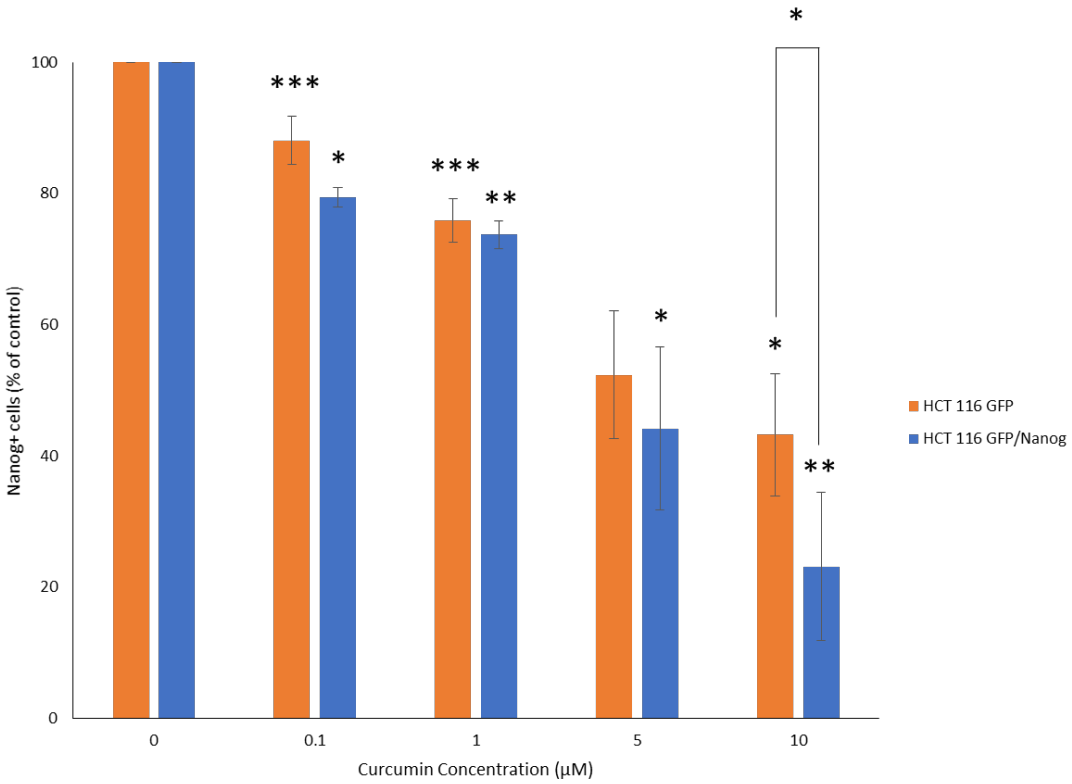


Figure 5-3. Effect of curcumin on Nanog^{+} cell population of HCT116^{GFP} and HCT116$^{GFP/Nanog}$ cells. Cells were plated as previously described. All values are expressed as a % relative of respective solvent control and mean±SEM of triplicate experiments (*p<0.05, *p<0.001, ***p<0.001 as determined by students T-Test).

Subsequently, the effect of curcumin on proliferation of $Nanog^+$ cells was explored. A significant reduction in $Nanog^+Ki67^+$ cell number was identified following curcumin treatment in both cell lines (20-70% reduction in sphere numbers for $HCT116^{GFP}$ and a 20-95% reduction in sphere number for $HCT116^{GFP/Nanog}$). In fact, $HCT116^{GFP/Nanog}$ cells appeared to be more sensitive to curcumin treatment at higher concentrations (5 and 10 μM) compared to $HCT116^{GFP}$ cells. This was significant between treatments only at 10 μM (see figure 5-4). Concurrently, though an increase in $Nanog^-Ki67^-$ expression is observed at 5 and 10μM treatments, it is non-significant (figure 5-4).

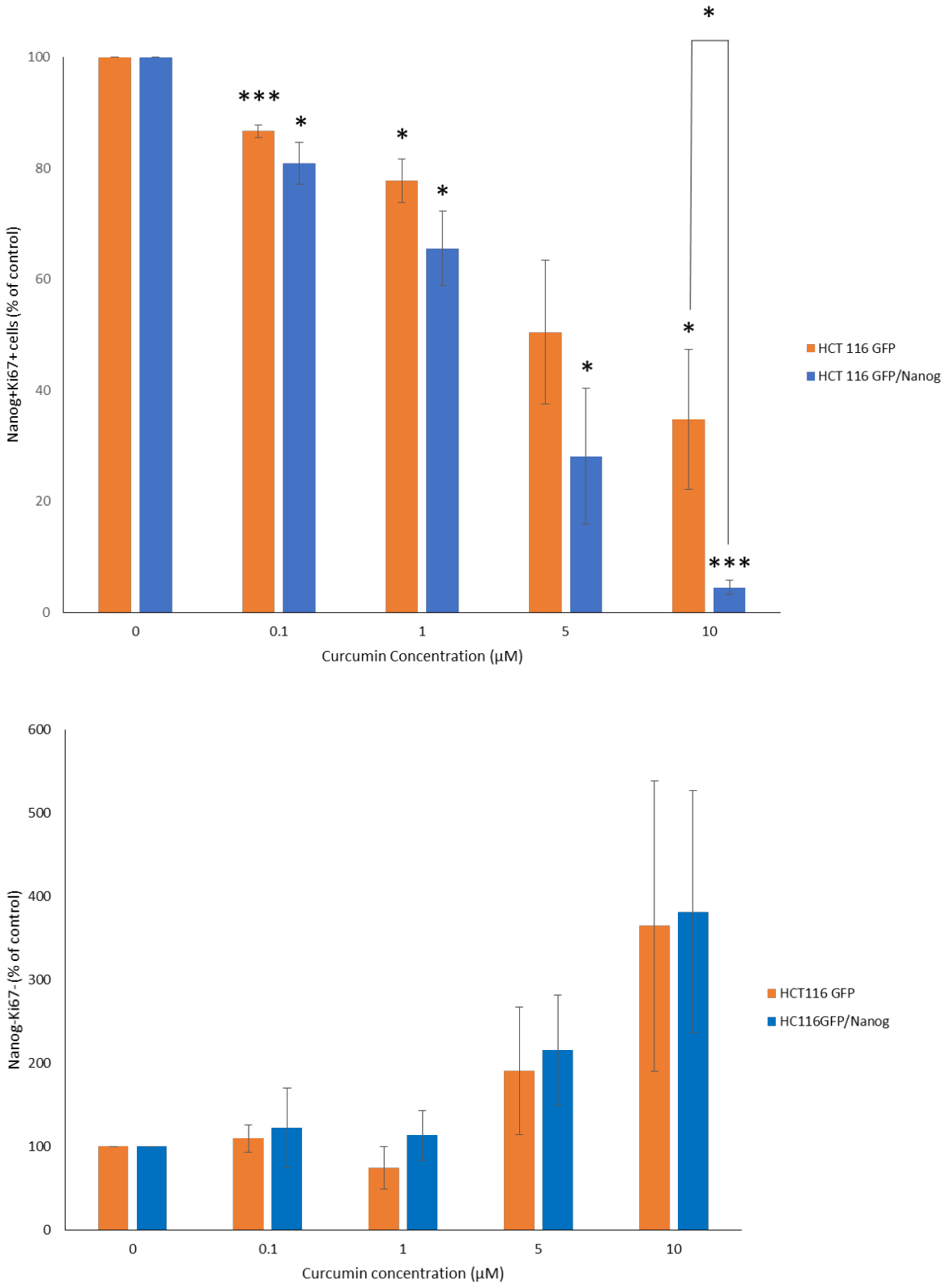


Figure 5-4. Effect of curcumin on Nanog^{+}Ki67^{+} and Nanog^{-}Ki67^{-} cell population of HCT116^{GFP} and HCT116 $^{GFP/Nanog}$ cells. Cells were plated as previously described. All values are expressed as a % relative of respective solvent control and mean±SEM of triplicate experiments (*p<0.05, *p<0.001, ***p<0.001 as determined by students T-Test).

In addition, the effect of curcumin on the non-CSC proliferating population ($Nanog^{-}Ki67^{+}$) was explored (figure 5-5). A clear increase in $Nanog^{-}Ki67^{+}$ cells was seen, particularly at low concentrations of curcumin treatment (0-5 μM) was apparent (although non-significant) in the $HCT116^{GFP/Nanog}$ cell line compared to the $HCT116^{GFP}$ cell line. In contrast to the increase in $Nanog^{-}Ki67^{+}$ cells seen following low concentrations of curcumin treatment, a decrease in $Nanog^{-}Ki67^{+}$ cells was observed at 10 μM in both cell lines (figure 5-5). It may be that at higher concentrations, curcumin induces apoptosis and cell death, whilst at lower concentration proliferation is affected. This is in keeping with previous published literature [224]. In addition, a more variable response is detected in $HCT116^{GFP}$ cells. This may be due to the differences in CSC and non-CSC population in culture. Though stem cells grow more readily in spheres, when analysing using FACS analysis, all cells within a well are collected and processed. As sphere cultures are used this should be relatively few.

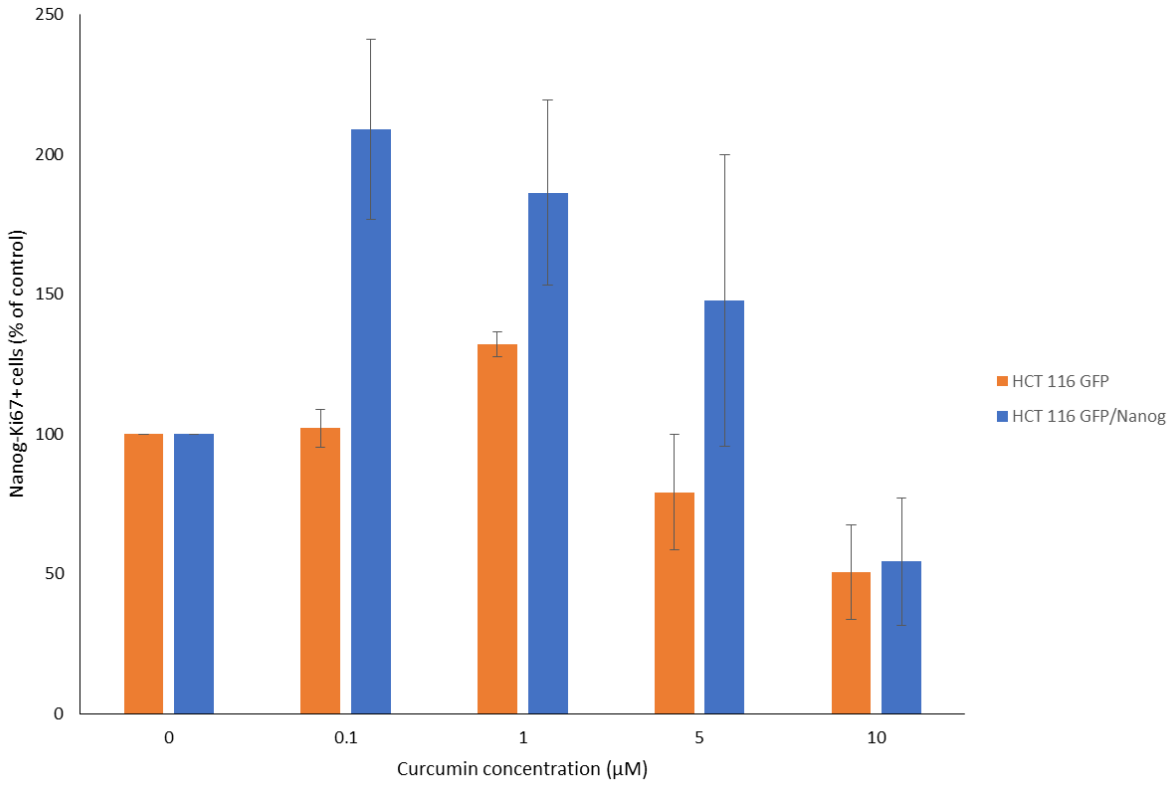
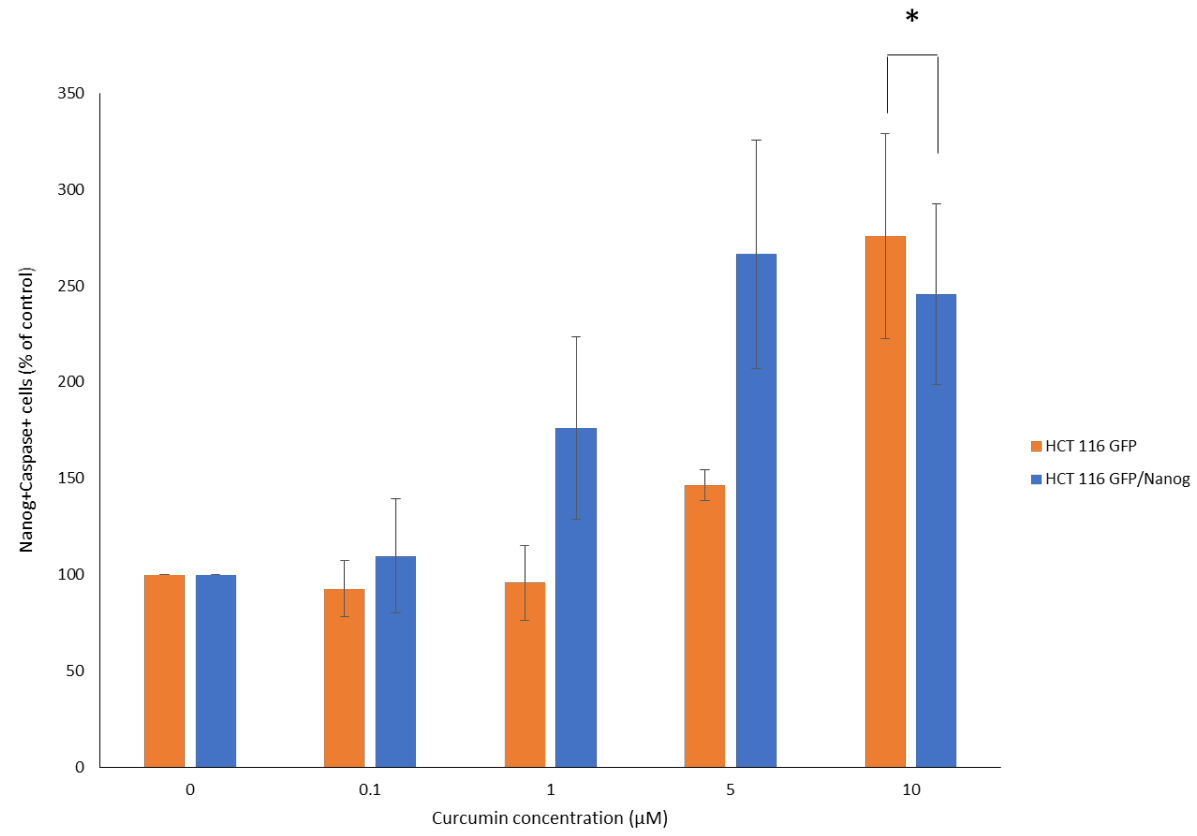


Figure 5-5. Effect of curcumin on $Nanog^{-}Ki67^{+}$ cell population of $HCT116^{GFP}$ and $HCT116^{GFP/Nanog}$ cells. Cells were plated as previously described. All values are expressed as a % relative of respective solvent control and mean±SEM of triplicate experiments (*p<0.05, *p<0.001, ***p<0.001 as determined by students T-Test).

Subsequently, the effect of curcumin on $Nanog^{+}Caspase^{+}$ cells was investigated to determine apoptosis-inducing effects of curcumin specifically on the CSC fraction (figure 5-6). Whilst curcumin induced cleaved caspase-3 in the $HCT116^{GFP/Nanog}$ cell line from 0.1 μM (from 110%-250%) and in the $HCT116^{GFP}$ cell line from 5 μM (150-250%), these increases were not significant. Curcumin is able to induce apoptosis in CRC CSCs has been previously published [225]. Furthermore, the $Nanog^{-}Caspase^{-}$ population was considered. An increase was observed, however this was non-significant.

Taken together these results suggest curcumin is able to target CSC and non-CSC populations. At low concentrations (<5 μM) treatment, curcumin is able to selectively induce apoptosis in cells expressing Nanog (non-significantly), however it is able to preferentially push cells into a non-stem fate significantly ($Nanog^{-}$). An important point to note is that the $HCT116^{GFP/Nanog}$ cell represent an artificial system that is being used to increase the magnitude of effect. It is important to ensure this hypothesis is tested in primary patient samples to evaluate the clinical utility of this. In addition, it may be that not all $Nanog^{-}$ cells are non-CSC - they may well express other markers indicative of stem-like activity.



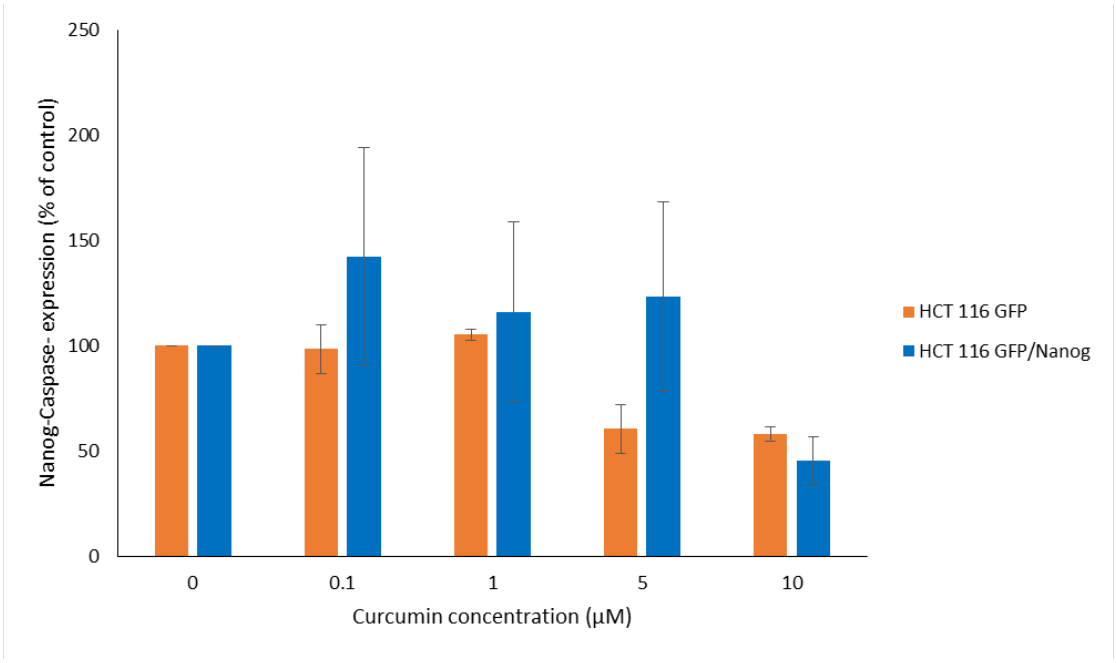


Figure 5-6. Effect of curcumin on Nanog^{+}Caspase^{+} and Nanog^{-}Caspase^{-} cell population of HCT116^{GFP} and HCT116 $^{GFP/Nanog}$ cells. Cells were plated as previously described. All values are expressed as a % relative of respective solvent control and mean±SEM of triplicate experiments (*p<0.05, *p<0.001, ***p<0.001 as determined by students T-Test).

5.4 Determining effect of curcumin on long-term sphere culture (>3 months) (3D)

There have been a number of concerns about the development of resistant clones as a result of curcumin treatment, in many types of cancer and CSCs [226]. To assess the potential for developing resistance, spheres were treated from both $HCT116^{GFP}$ and $HCT116^{GFP/Nanog}$ cell lines, harvested, replated as single cells and allowed to re-form spheres (see figure 5-7 and 5-8). They were not re-treated during this time allowing assessment of self renewal. Sphere size was assessed at 2 weeks (figure 5-9). Spheres which had been treated with curcumin at the highest concentration (10μM) were smaller than solvent control. When spheres were present in all wells they were treated for a second time. Following this, spheres were left intact in curcumin/solvent containing media. Spheres were again allowed to form in all wells and treated again for a third time. Images were taken periodically.

$HCT116^{GFP/Nanog}$ cells consistently had a greater sphere forming ability compared to $HCT116^{GFP}$ cells across 3 treatments. In addition they appeared to be more sensitive to curcumin treatment. There did not appear to be signs of resistance as following each of the treatments, spheres remained sensitive to treatment based on sphere forming capacity. This was not formally assessed. After leaving spheres in solvent containing media and then treating on alternate days, cell viability noticeably dropped therefore, following the 3rd treatment of curcumin, effect on sphere forming ability and resistance could not be assessed.

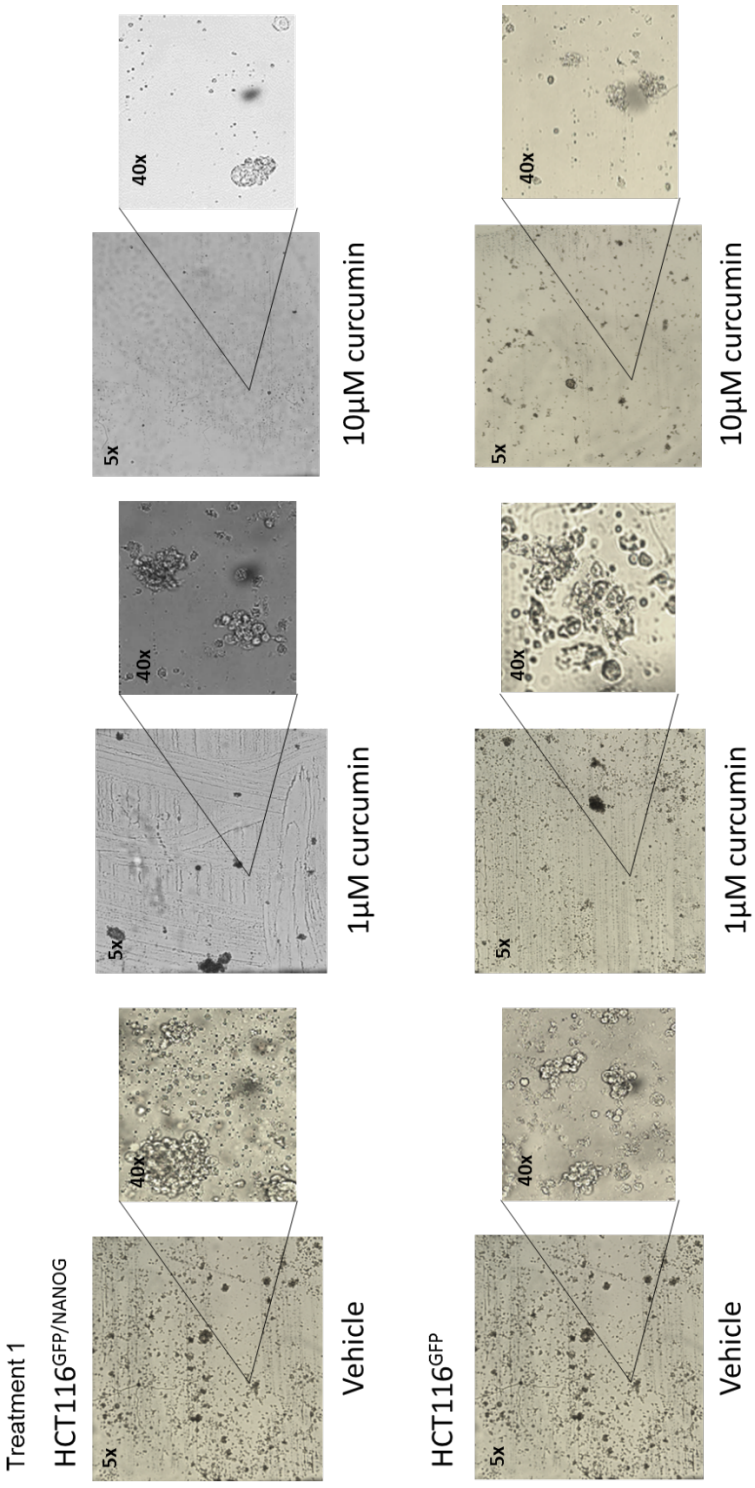
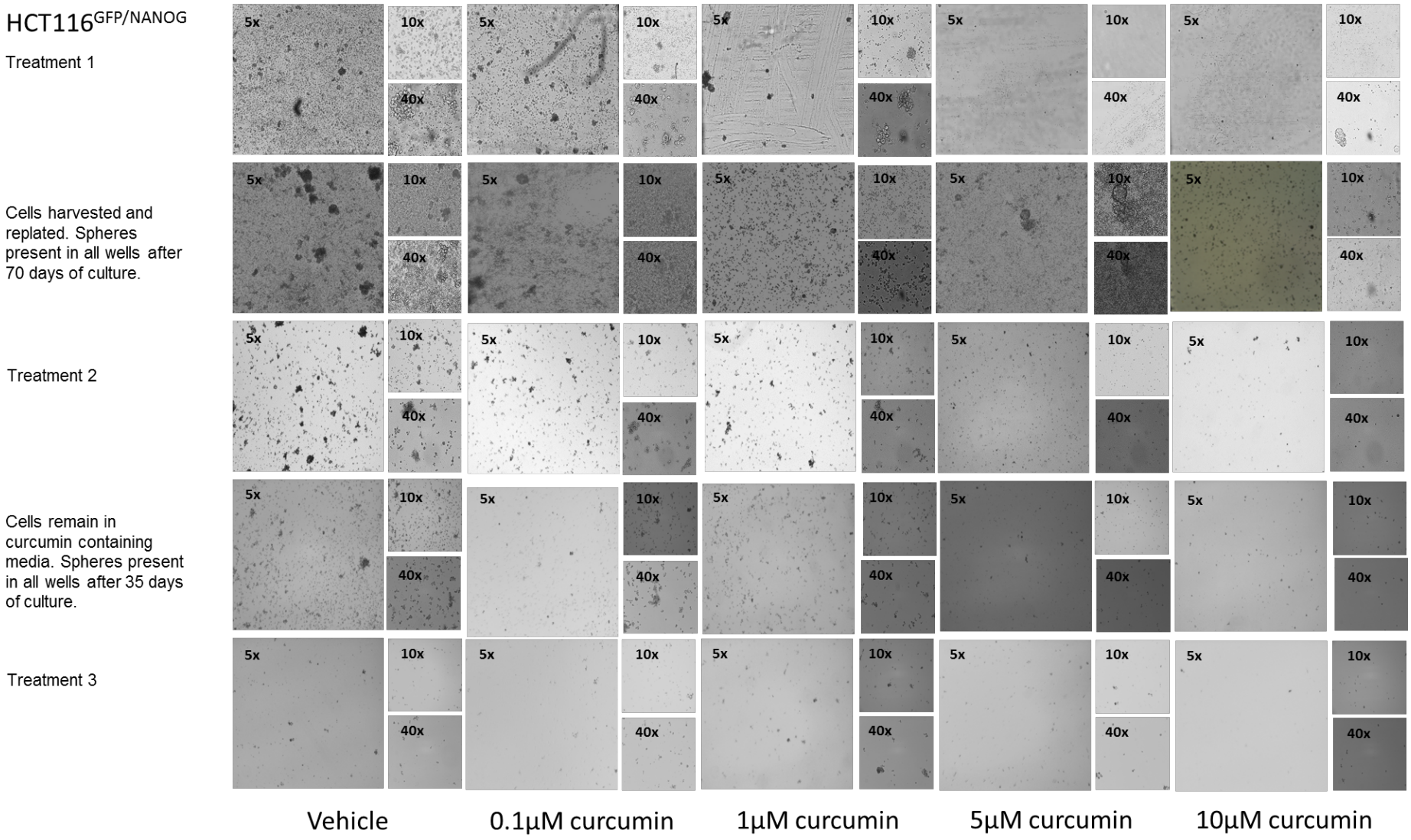


Figure 5-7. Effect of curcumin on sphere formation of HCT 116^{GFP} and HCT 116 $^{GFP/Nanog}$ cells. Single cells (1000/well) were plated, treated with curcumin on alternate day for 7 treatments (1st treatment). Representative images are shown.


HCT116GFP/NANOG
Treatment 1
Cells harvested and replated. Spheres present in all wells after 70 days of culture.
Treatment 2
Cells remain in curcumin containing media. Spheres present in all wells after 35 days of culture.
Treatment 3
5x
10x
40x
Vehicle
0.1µM curcumin
1µM curcumin
5µM curcumin
10µM curcumin

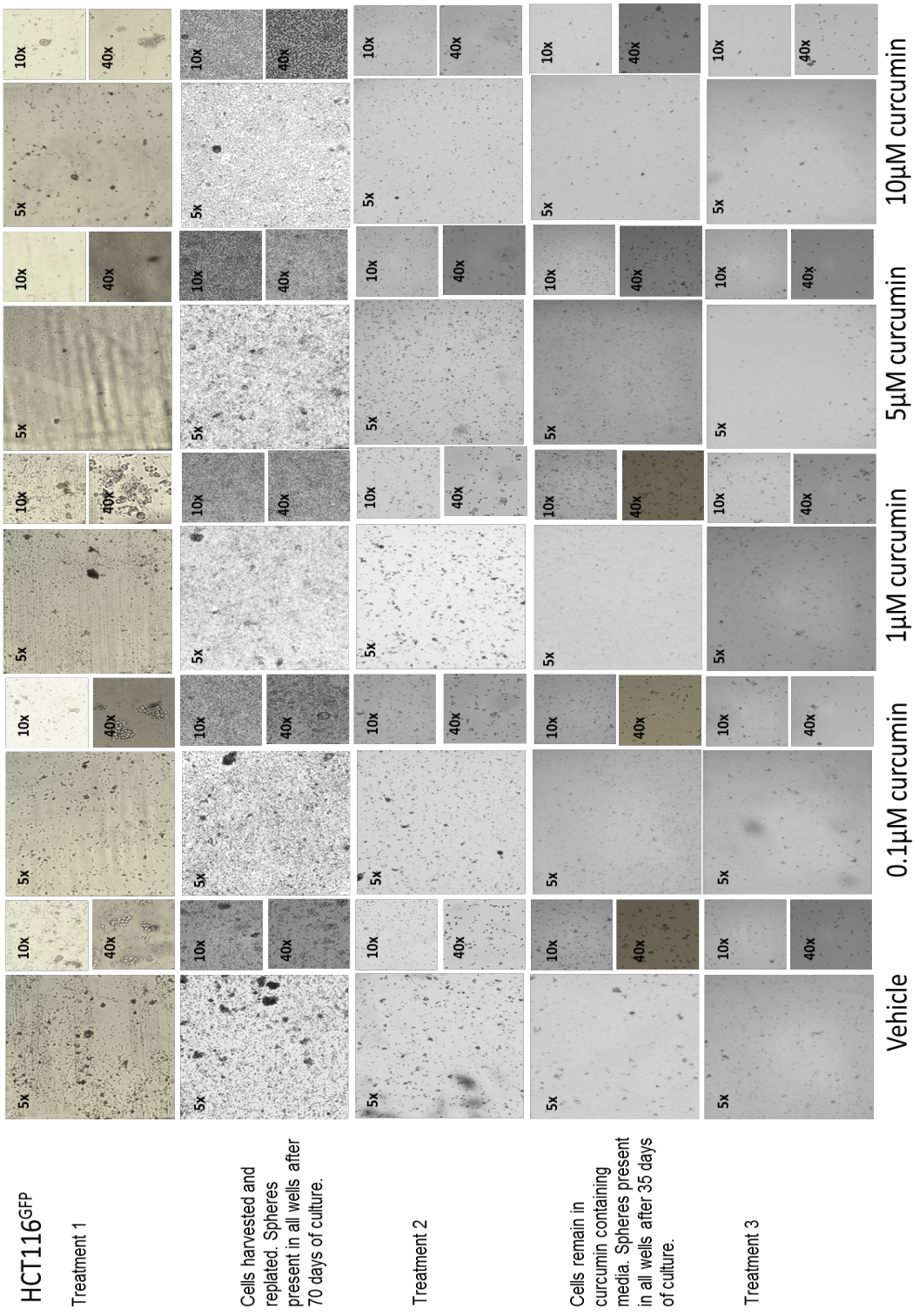


Figure 5-8. Effect of curcumin on sphere formation of HCT 116^{GFP} and HCT 116 $^{GFP/Nanog}$ cells. Single cells (1000/well) were plated, treated with curcumin on alternate day for 7 treatments (1st treatment). Following treatment spheres were harvested, single cells created and replated (1000 cells/well) in sphere medium. No curcumin was added. Cells were monitored for a period of 70 days till sphere were present in all wells. Cells were treated with curcumin on alternate days for 5 treatments (2nd treatment). Spheres were not harvested, but left in curcumin/solvent containing media. They were monitored for a further 30 days and retreated with curcumin (3rd treatment).

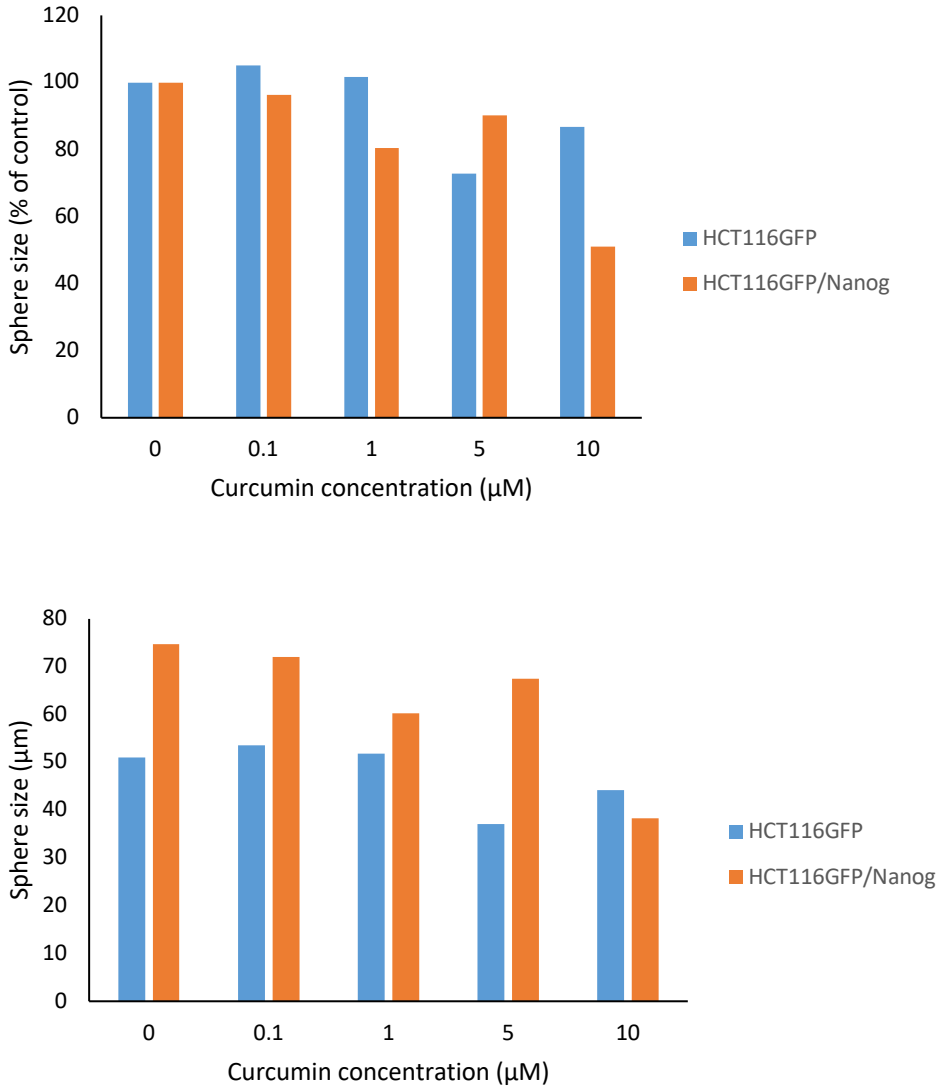


Figure 5-9. Effect of curcumin on sphere formation of HCT 116^{GFP} and HCT 116 $^{GFP/Nanog}$ cells. Single cells (1000/well) were plated, treated with curcumin on alternate day for 7 treatments (1st treatment). Following treatment spheres were harvested, single cells created and replated (1000 cells/well) in sphere medium. No curcumin was added. Cells were monitored for a period of 14 days and size assessed.

5.5 Effect of curcumin on Nanog protein stability (2D)

Emetine and cycloheximide are inhibitors of protein synthesis. They are often used to help determine the half-lives of proteins in cultured cells. The effect of curcumin on Nanog stability in HCT116$^{GFP/Nanog}$ cells was assessed with and without 0.1 μM curcumin treatment and either emetine or cycloheximide over a period of 0-6 hours. Following curcumin treatment, Nanog stability was not significantly affected (figure 5-10 as representative example).

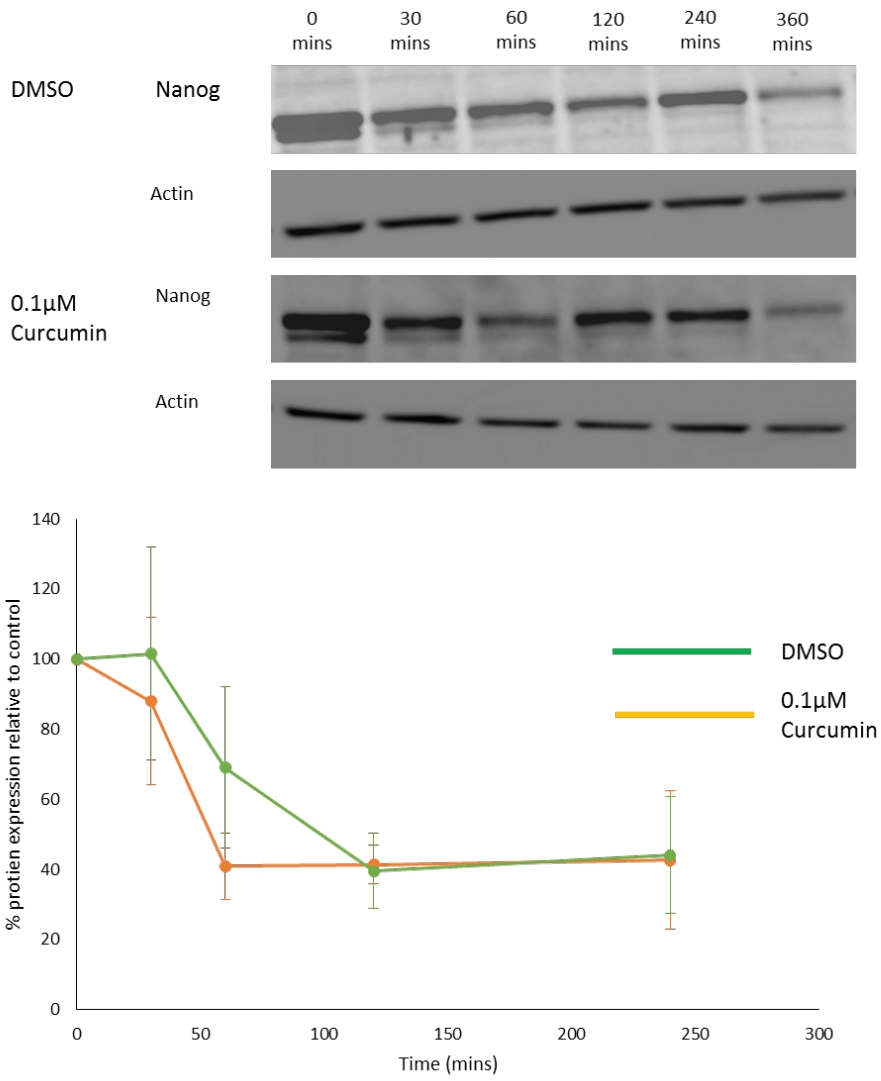


Figure 5-10. Emetine assay for assessing stability of Nanog. Representative western blots following emetine or cycloheximide exposure in HCT116$^{GFP/Nanog}$ cells and densitometry analysis. Cells were treated with emetine (20μg/mL) and either solvent or 0.1μM curcumin. Cells were harvested at a range of different time points. Actin was used to normalise the data. Values are representative of mean±SEM of quadruplet independent experiments.

5.6 Conclusion

Established cell lines in the pre-clinical assessment of potential anti-cancer drugs have been extensively used across virtually all types of malignancy for decades [223]. However, in many instances there is a poor correlation between the efficacy of candidate anti-cancer drugs observed in primary patient cells or clinically, and activity demonstrated in cell lines [227-231]. Despite this cell lines are frequently used, as they are readily available, inexpensive to maintain and provide some insight into drug activity. Problems include a lack of heterogeneous populations and clonal evolution, particularly when considering early cancerous changes and premalignant changes in humans. This is particularly apparent for genetically modified cell lines.

Nevertheless, these types of cells are extremely important in in delineating mechanisms of interest and cell behaviour which may be difficult to capture in human tissues. Additionally, candidate biomarkers can be established in cell lines and then reassessed in human tissues. One of the major differences when using cell lines is their higher rate of proliferation compared to cancer cells *in vivo* [232, 233]. Tumour cells *in vivo* exhibit a much lower proliferation rate than *in vitro* cell lines, which are often selected for rapid growth with doubling times much shorter than the cancer cells *in vivo* [232]. This is an important consideration as within this chapter, cellular proliferation of cells was used as a marker of curcumin's efficacy which will require further validation in human tissues and primary cells.

It is important to note, though $HCT116^{GFP/Nanog}$ cells were more sensitive to curcumin treatment than $HCT116^{GFP}$ cells, this does not demonstrate a clear mechanism of action. As a result, more quantitative analysis will be carried out using RNASeq and possibly CHIPSeq experiments in the future. This will allow an understanding of the gene expression and protein expression changes as a result of curcumin treatment, in a population of cells which are enriched for Nanog expression. Important or dominant changes can then be verified in human tissues and Caco2 cells.

Based on this work, it would be important to assess $Nanog^{+}$ and $Nanog^{+}Ki67^{+}$ expression in human explant tissues. This will be considered in the next chapter. In addition, $Nanog^{+}Caspase^{+}$ could also be considered. However, it is likely there are high levels of apoptosis present in human cancer tissues following 2 days of culture, as the tissue culture environment is unlikely to provide all nutrients required for tissue viability.

The data presented in this chapter suggests curcumin does not have an effect on Nanog stability. Therefore, an alternative hypothesis needs to be considered for example CSC plasticity.

6.0 Chapter 6 (Results and Discussion) – Effect of curcumin on 3D human explant adenoma and CRC tissues

6.1 Introduction

For many years the use of patient-derived xenografts in murine studies has been the gold standard in testing efficacy of pharmacological interventions. These have led to the effective translation of many compounds to a clinical platform, resulting in an increase in the use of PDX models [197]. Indeed, these models are becoming more readily available via commercial routes. There are several advantages to PDX models. These include their ability to retain the original tissue architecture of the human sample, as well as the heterogeneity of the tumour. In addition, injecting orthotopically can lead to more information regarding pharmacokinetics and pharmacodynamics assessments, ensuring tissues are targeted appropriately [234, 235]. However a bias exists in that certain tumour types are more likely to engraft that others, resulting in greater PDX resource for some tumour types than others. In addition, the immune response is also void in many PDX models, and there is increasing evidence this plays a crucial role, particularly in cancer therapy. When immune systems have been produced in humanised mouse models these have not always shown similar responses in a human setting. In addition, the stromal components from human tumours, are often replaced by murine components, hindering the ability to assess the role of these components in pharmacological therapies [236].

Alternative approaches to test drug efficacy include the use of primary human tissue models [237-239] or cell based culture systems [240, 241]. There are two main types of primary tissue models: the ‘top down’ approach, where the tissue integrity is maintained and cultured as a tumour slice culture or explant microtissues or the ‘bottom up’ method, where the architecture of a tissue is recreated using scaffolds and multiple types of cells. Though these approaches are not well established in all groups, and challenges exist regarding set up and a move from murine models, significant progress has been made. In the work presented here, a top down approach using human colorectal cancer tissues, has been used to assess the potential efficacy of curcumin in a range of colorectal cancer subtypes [197].

6.2 Demographics (flow cytometry using an intracellular staining based assay)

Demographics of the patient samples used for 3D explant cultures are shown in table 6-1. There is a higher proportion of samples from males compared to females, as reflected in the general population with a mean age of 68 in men and 57 years in women. A range of cancer stages was collected and a higher number of left sided (12) compared to right sided (8) cancers were collected, which is reflected nationally. Similar figures were seen for the patient samples which were used to assess baseline CSC expression.

Table 6-1. Demographics of samples used for explant culture and intracellular staining ($Nanog^+$/$ALDH1A1^+$ and $Ki67^+$ expression).

Gender	Male	Female
n	14	6
Mean age at diagnosis	68 years	57 years
Range	48-82 years	35-70 years
Cancer Stage		
1	3	1
2	4	1
3	4	3
4	0	1
Site of cancer		
Left sided cancer	10	2
Right sided cancer	4	4
Adenomas	3	0

6.3 Gating strategy

Tissues were obtained, cubed and treated with various doses of curcumin (0-10 μM) for 24 hours. Subsequently, they were processed into single cells, fixed and permeabilised for intracellular staining. The gating strategy and example flow cytometry plots were as previously described. Each patient tissue was treated with 4 different concentrations of curcumin as a single replicate. This was due to limitations in tissue obtained following resection.

6.4 Nanog$^+$ expression in baseline CSC profile samples vs explant colorectal tissues

Baseline Nanog$^+$ expression following 24 hours of vehicle treatment, ranged from <0.5% in 5 samples, 0.5%-1.0% in further 8 samples and >1.0%-25% in the remaining 7 samples. This is different to the samples used for baseline profiling where, baseline Nanog$^+$ expression was <0.5% in 1 sample (stage 1), >0.5-1.0% in 4 samples and >1.0%-35% in the remaining 41 samples. Thirteen of the patient tissues used in the explant studies were also profiled for baseline Nanog expression. Tissues had significantly higher levels of Nanog at baseline CSC profiling compared to after explant culture (see figure 6-1). This could be for a range of reasons including effects of vehicle (DMSO) and effects of explant culture on CSC expression and tissue integrity following 24 hours acclimatisation and 24 hours treatment [242]. It is unlikely to be as a result of tissue processing, as both the baseline samples and explant samples were treated in a similar way. An example of H and E's of explant tissues treated with curcumin is also shown (figure 6-2).

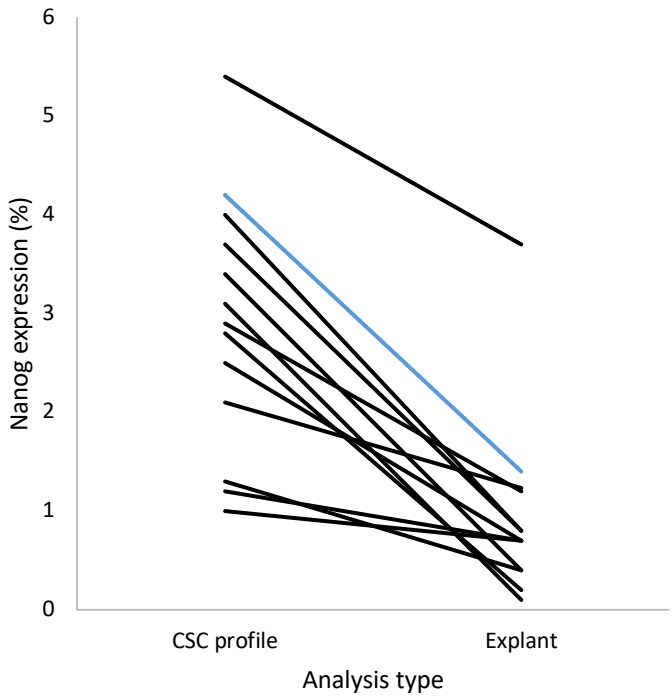


Figure 6-1. Effects of explant culture on Nanog$^+$ expression. Nanog$^+$ expression was assessed at baseline, when samples were obtained. In summary, these samples were obtained from theatre, processed into single cells and kept at -80°C til analysis. Tissues used for explant culture, were cubed, allowed to acclimatise for 24 hours and treated with curcumin for a further 24 hours. Subsequently, they were processed into single cells and analysed. Nanog$^+$ levels were significantly higher in CSC profiled samples, than paired samples used for explant culture ($P<0.001$ paired students T test).

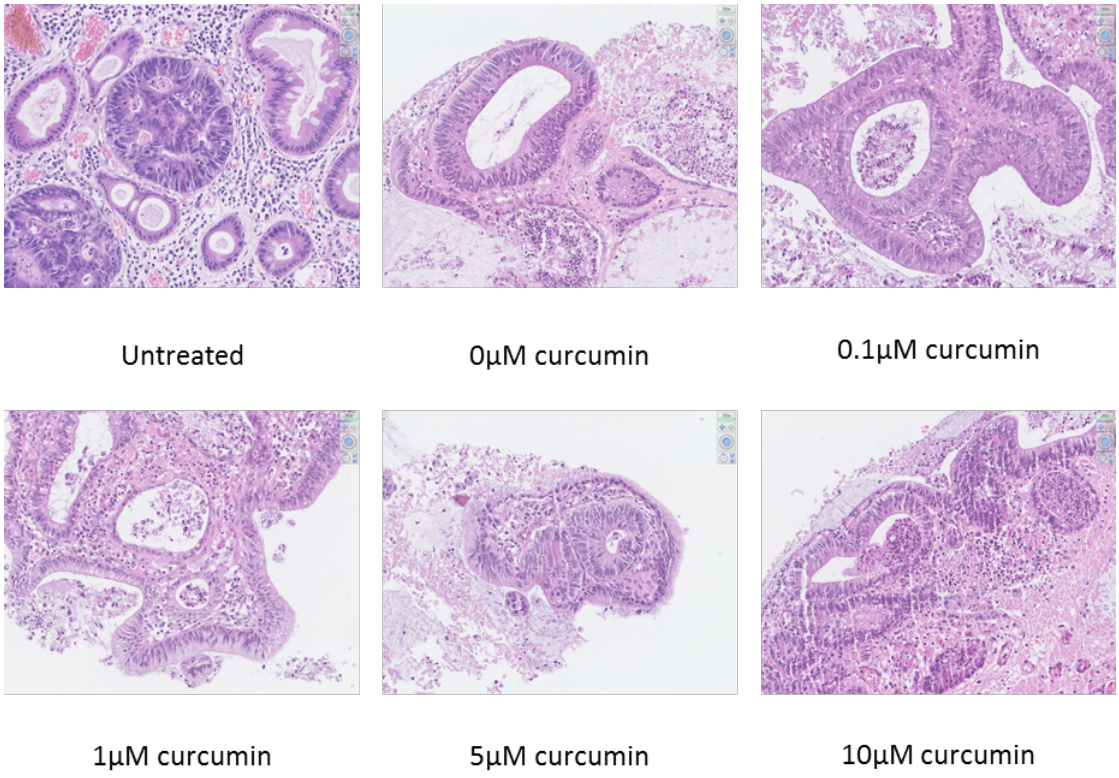


Figure 6-2. Example H and E's of explant tissues. Following 24 hour treatment with curcumin the morphology and integrity of tissues was maintained (x20).

6.5 Investigating the effect of curcumin treatment on Nanog⁺ expression in CRC explant tissues

To consider the effect of curcumin on Nanog⁺ expression in a limited supply of tissue, each sample was treated with 4 concentrations. This allowed a range of concentrations to be assessed in each patient (figure 6-3). No significant changes in Nanog⁺ expression were identified. The maximum increase or reduction in Nanog⁺ expression is shown (figure 6-3). A >35% maximum reduction in Nanog⁺ expression was seen in 16/20 samples assessed. There appears to be an increase in Nanog⁺ expression in 3 patient samples. On closer examination, in 1 patient sample, at a single concentration, a higher expression of Nanog⁺ was detected, whilst at all other concentrations including control, similar levels were detected. In the remaining 2 patient explants, lower levels of Nanog⁺ were detected in control samples, compared to all curcumin samples tested, where Nanog⁺ expression was similar, leading to an apparent increase (see figure 6-4). This could partly be explained by heterogeneity within the tumour. When tumours were cubed prior to explant culture, care was taken to ensure a variety of tumour areas were distributed to each treatment well. Discrepancies in this may lead to certain wells not having a representative tumour population. To consider this further, where a decrease

in Nanog$^+$ expression was detected, it was detected in 3 or 4 of the concentrations tested in 75% of samples tested (n=15/20 samples). In the remaining samples, 4 had no response and in 1 sample a reduction in Nanog$^+$ expression was detected in 2 of the concentrations assessed. In some patient tissues no response was detected (n=4), in others a traditional linear dose response was seen (n=7) and in the remaining samples a non-linear response was observed (see figure 6-3). The effect of curcumin on Nanog$^+$ expression at each concentration is shown in figure 6-5. A single concentration did not exhibit a greater effect on Nanog$^+$ expression compared to another. Though in some patient tissues a U-shaped response was detected i.e. a greater response seen at lower concentrations (0.1-5 μM), than in 10 μM. Where a linear response is seen, it may be that the point at which a U-shaped response would be observed has not yet been reached.

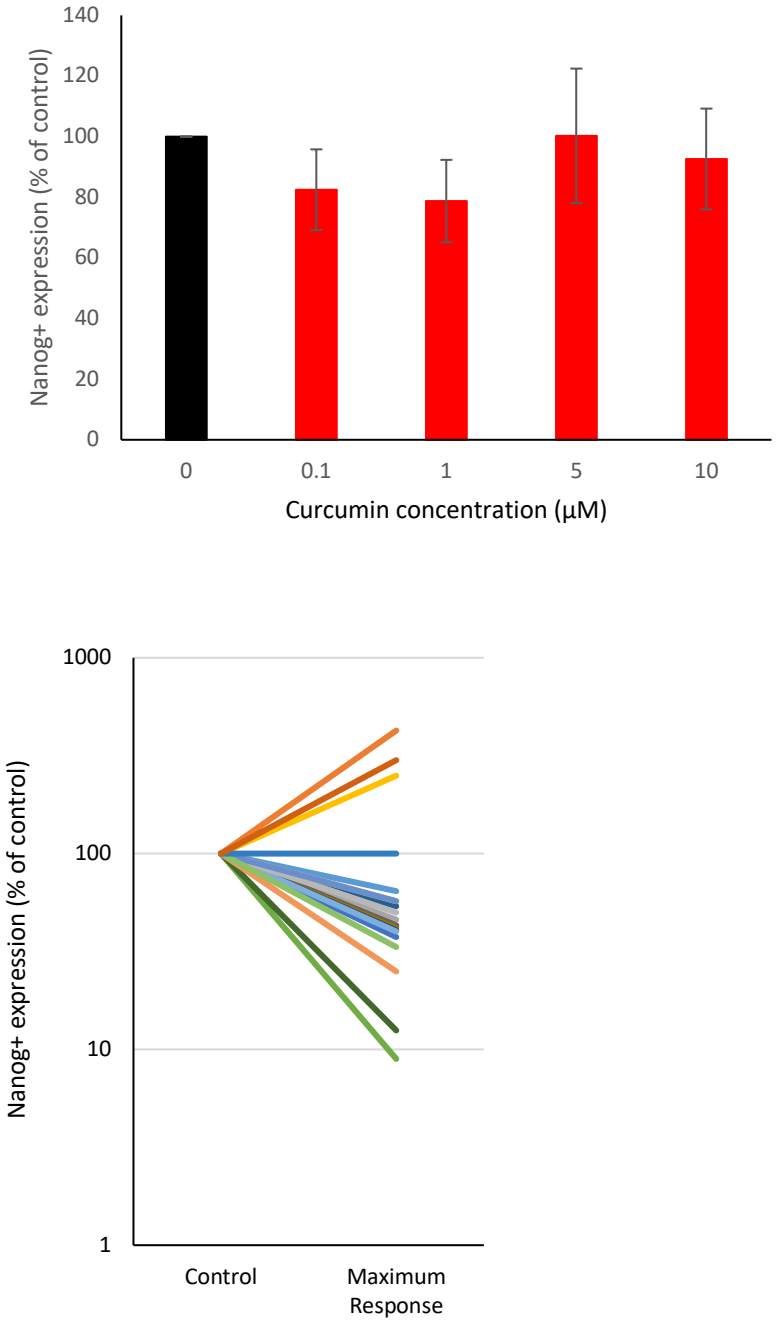


Figure 6-3. Curcumin does not alter Nanog$^+$ expression in primary colorectal tissues. Primary CRC tissues were cubed, allowed to acclimatise in explant media overnight and treated with curcumin for a further 24 hours at 0, 0.1, 1, 5 and 10 µM. Tissues were harvested, minced and single cells created using collagenase. Subsequently, cells were fixed and permeabilised, antibodies added and analysis carried out. The effect of curcumin on Nanog$^+$ expression (top) and greatest increase or decrease in Nanog$^+$ expression is shown (below). Results from a single replicate are shown from 20 patient samples.

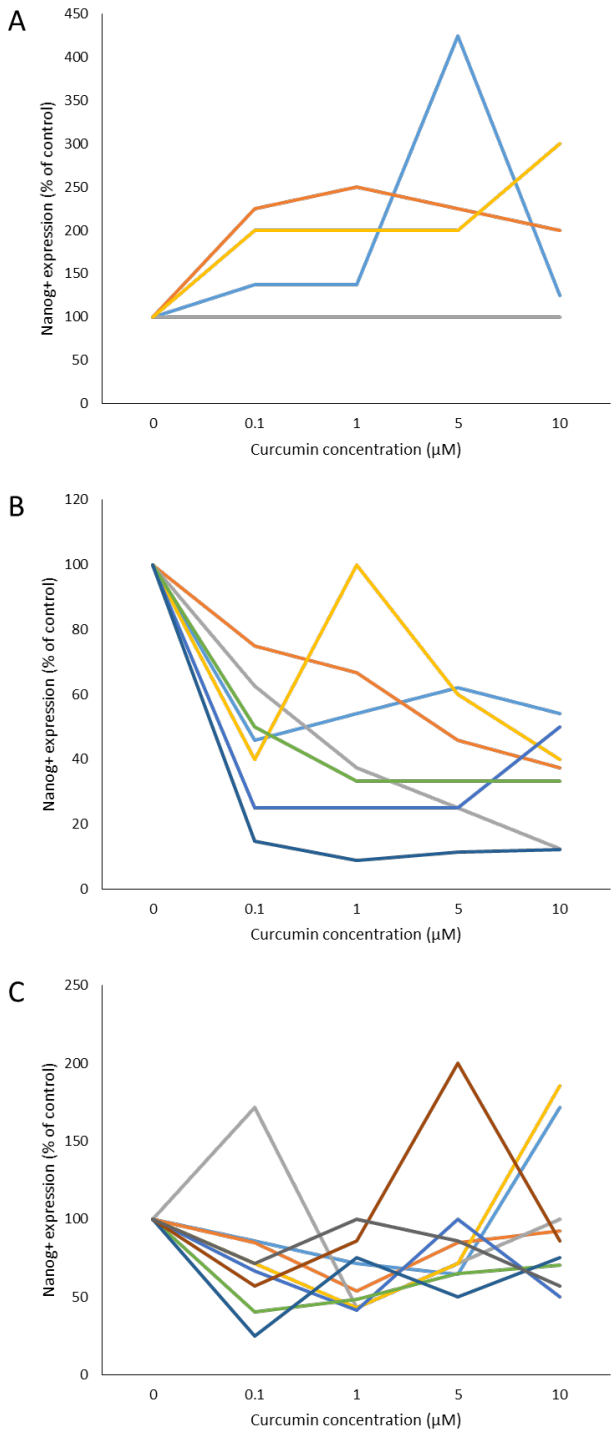


Figure 6-4. Effect of curcumin on Nanog$^+$ expression in primary colorectal tissues. Primary CRC tissues were cubed and treated with curcumin for 24 hours. Nanog$^+$ expression was assessed. A range of responses were seen. (A) No response (B) Linear treatment response (C) Non-linear response. Results from a single replicate are shown.

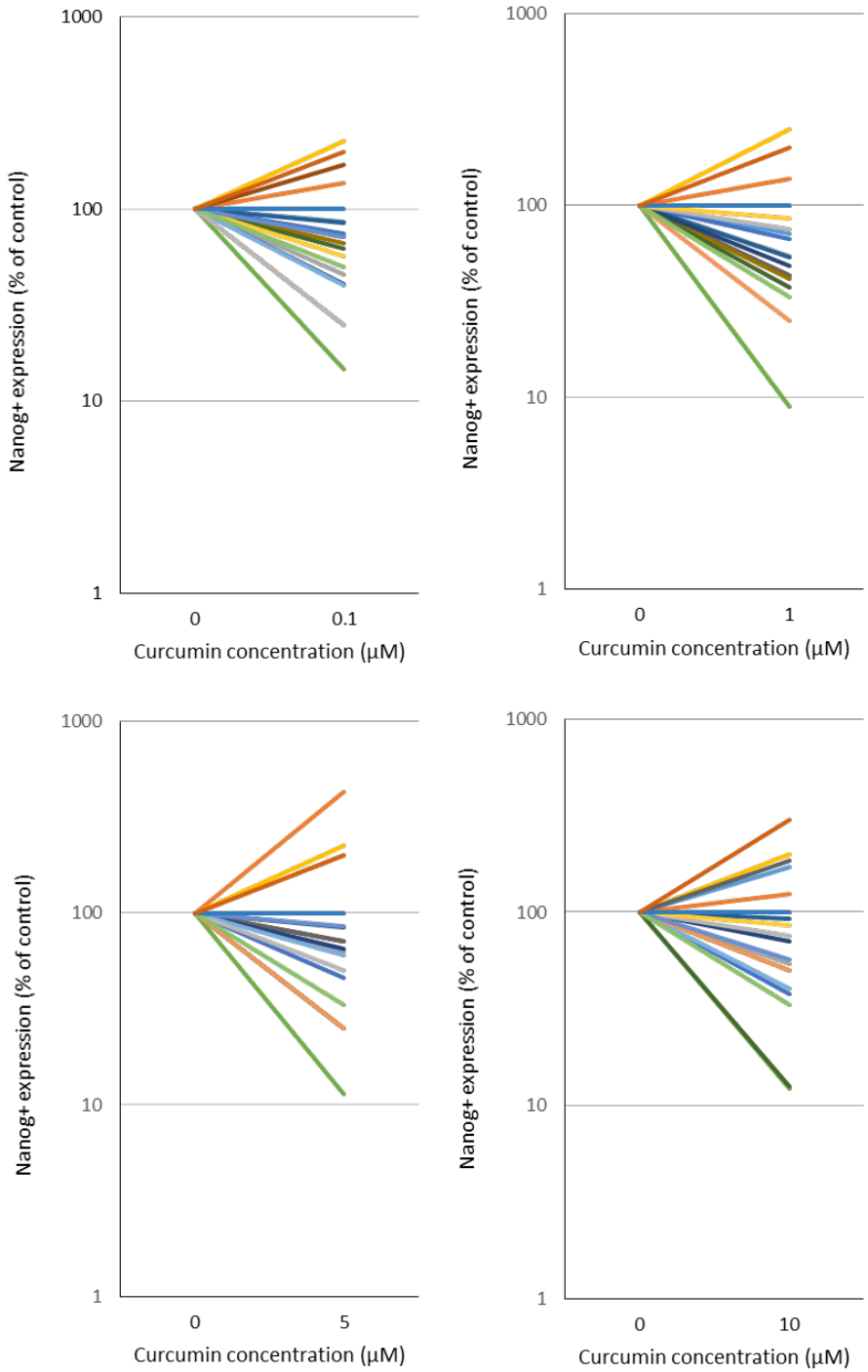


Figure 6-5. Curcumin has no effect on Nanog$^+$ expression in primary colorectal tissues. Primary CRC tissues were cubed, cultured in explant media overnight and treated with curcumin for a further 24 hours (0-10 µM). Tissues were harvested, minced and single cells created obtained. Subsequently, cells were fixed and permeabilised, antibodies added and analysis carried out. Results from a single replicate are shown.

6.6 Investigating the effect of curcumin treatment on Nanog$^+$Ki67$^+$ and Nanog$^-$Ki67$^-$ expression in CRC explant tissues

Following curcumin treatment change in Nanog$^+$Ki67$^+$ was assessed (figure 6-5). A significant decrease following treatment with 0.1μM and 1μM curcumin was observed. In 15/20 patient tissues a ≥50% reduction in Nanog$^+$Ki67$^+$ expression was observed. Therefore, a greater effect on Nanog$^+$Ki67$^+$ expression was detected in tissues compared to Nanog$^+$ alone (see figure 6-6). In 6 patient tissues following curcumin treatment <1% Nanog$^+$Ki67$^+$ expression was detectable. This suggests curcumin is able to target Nanog$^+$ expressing cells which are proliferating within 24 hours of treatment. In the 4 patient samples which did not show a reduction in Nanog$^+$ expression, a reduction in Nanog$^+$Ki67$^+$ was also not observed (see figure 6-7). In 1 patient tissue, similar levels of Nanog$^+$Ki67$^+$ were observed in control and all wells treated with curcumin, with the exception of one well. This may be due to tumour heterogeneity or experimental error. In the remaining samples a linear dose response (n=7) or a non-linear response was observed (n=8) (see figure 6-6). Where a linear response was seen, a decrease in expression was observed in all 4 concentrations except one tissue sample. Where a non-linear response was seen, a reduction in expression levels were seen 3 concentrations tested, in all but 2 samples. For the remaining samples, in 1 sample a reduction was seen in all 4 concentrations tested, and in the other sample, a reduction was seen at 2 concentrations. The effect of curcumin on Nanog$^+$Ki67$^+$ expression at each concentration is shown in figure 6-8. One concentration did not exhibit a greater effect on Nanog$^+$Ki67$^+$ expression compared to another. Lastly, the effect of curcumin on Nanog$^-$Ki67$^-$ cells was assessed. No significant changes were observed (figure 6-9).

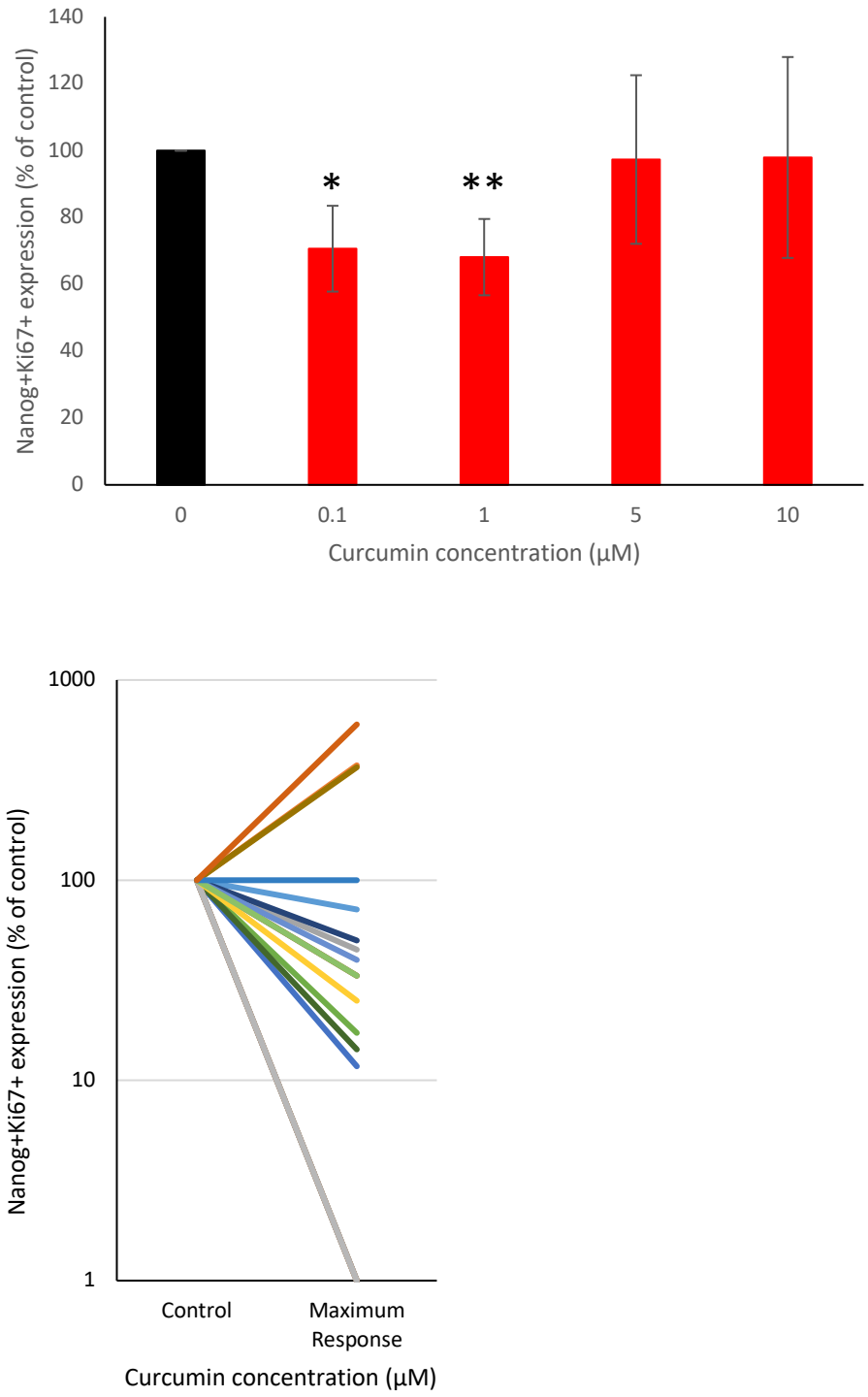


Figure 6-6. Curcumin reduces Nanog^{+}Ki67^{+} expression in primary colorectal tissues. Primary CRC tissues were cubed and treated with curcumin for 24 hours (0-10 µM). Tissues were harvested, minced and single cells created using collagenase. Subsequently, cells were fixed and permeabilised, antibodies added and analysis carried out. The effect of curcumin on Nanog^{+}Ki67^{+} expression (top) and greatest increase or decrease in Nanog^{+}Ki67^{+} expression is shown (below). Results from a single replicate are shown from 20 patient samples. (*p<0.05 and **p<0.001as determined by students T-Test).

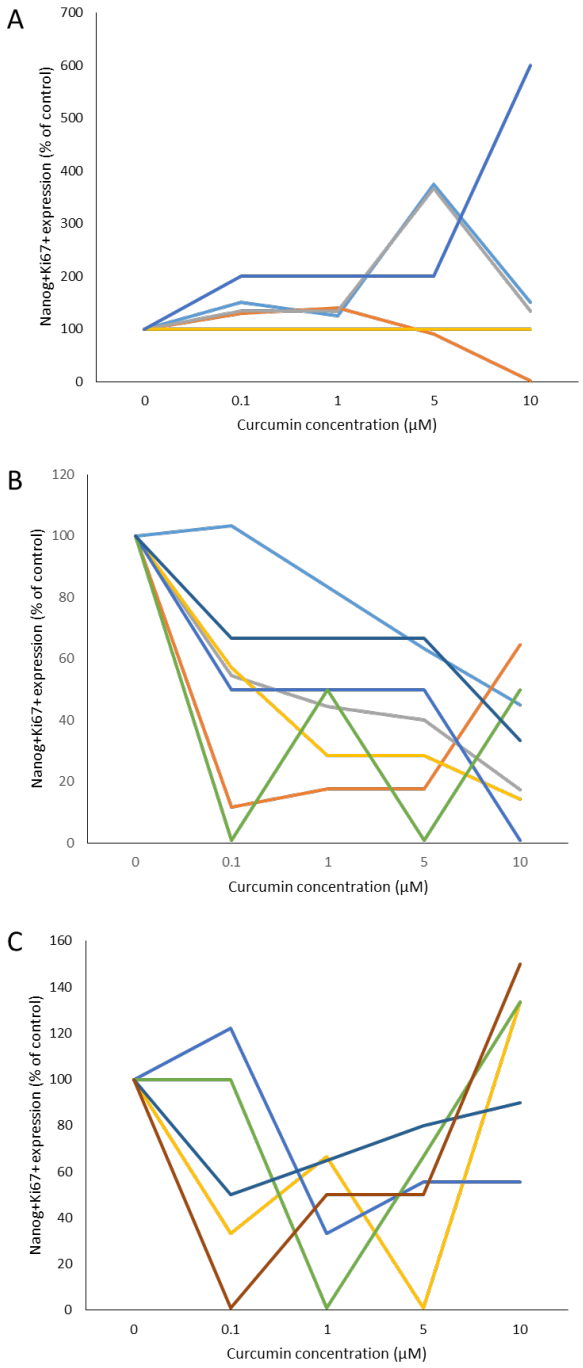


Figure 6-7. Effect of curcumin on Nanog^{+}Ki67^{+} expression in primary colorectal tissues. Primary CRC tissues were cubed and treated with curcumin for 24 hours. Nanog^{+}Ki67^{+} expression was assessed. A range of responses were seen. (A) No response (B) Linear treatment response (C) Non-linear response. Results from a single replicate are shown.

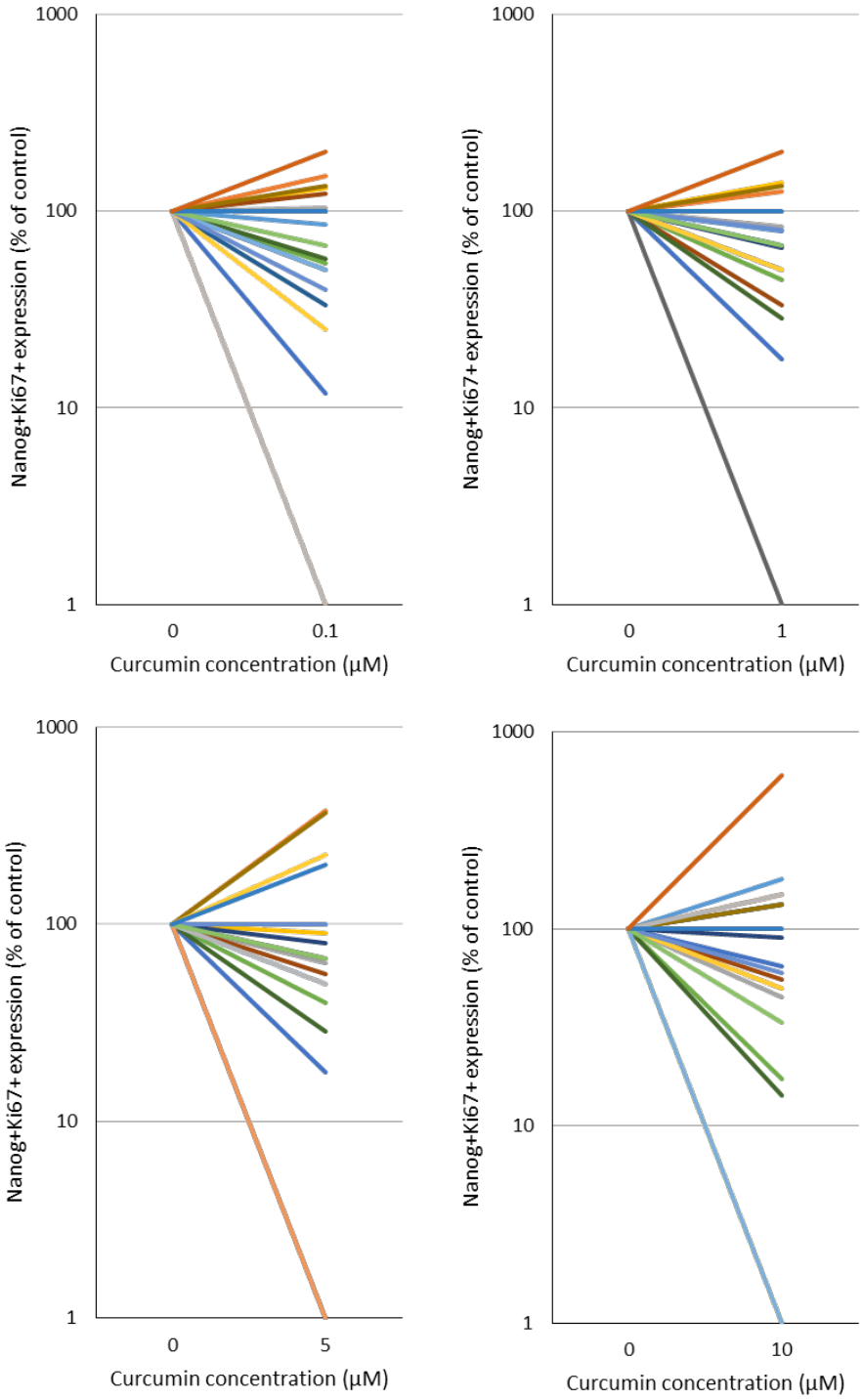


Figure 6-8. Curcumin reduces Nanog^{+}Ki67^{+} expression in primary colorectal tissues. Primary CRC tissues were cubed, cultured in explant media overnight and treated with curcumin for a further 24 hours (0-10 µM). Tissues were harvested, minced and single cells created obtained. Subsequently, cells were fixed and permeabilised, antibodies added and analysis carried out. Results from a single replicate are shown.

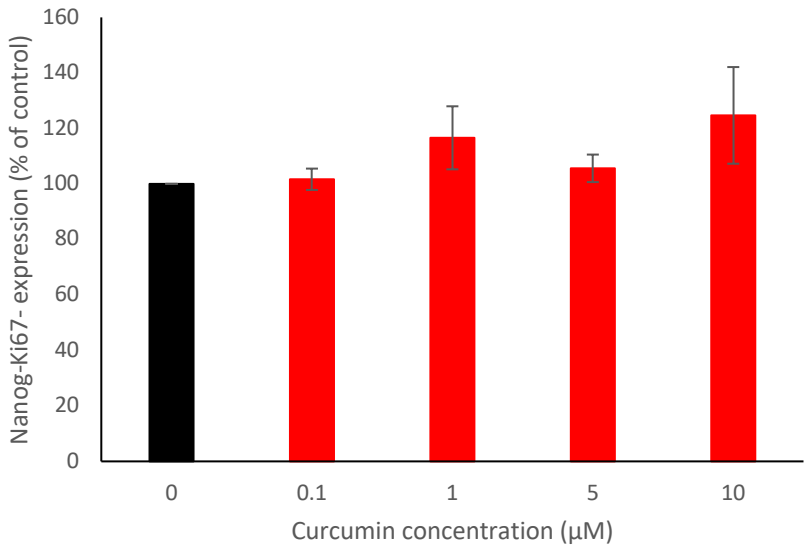


Figure 6-9. Curcumin does not alter Nanog^{-}Ki67^{-} expression in primary colorectal tissues. Primary CRC tissues were cubed and treated with curcumin for 24 hours (0-10 µM). Tissues were harvested, minced and single cells created using collagenase. Subsequently, cells were fixed and permeabilised, antibodies added and analysis carried out. Results from a single replicate are shown from 20 patient samples.

6.7 Investigating the effect of curcumin treatment on Nanog^{+}ALDH1A1^{+} expression in CRC explant tissues

Following curcumin treatment, in 13/18 patient tissues a ≥50% decrease in Nanog^{+}ALDH1A1^{+} expression was observed, suggesting double positive cells can be targeted with curcumin treatment (see figure 6-10). This was non-significant. No response as detected in 4 patient tissues. These were the same tissues where a response to curcumin treatment was not observed in Nanog^{+} or Nanog^{+}Ki67^{+} populations. In addition, similar trends were observed regarding those who responded in a dose dependent or non-linear fashion (see figure 6-11 and 6-12).

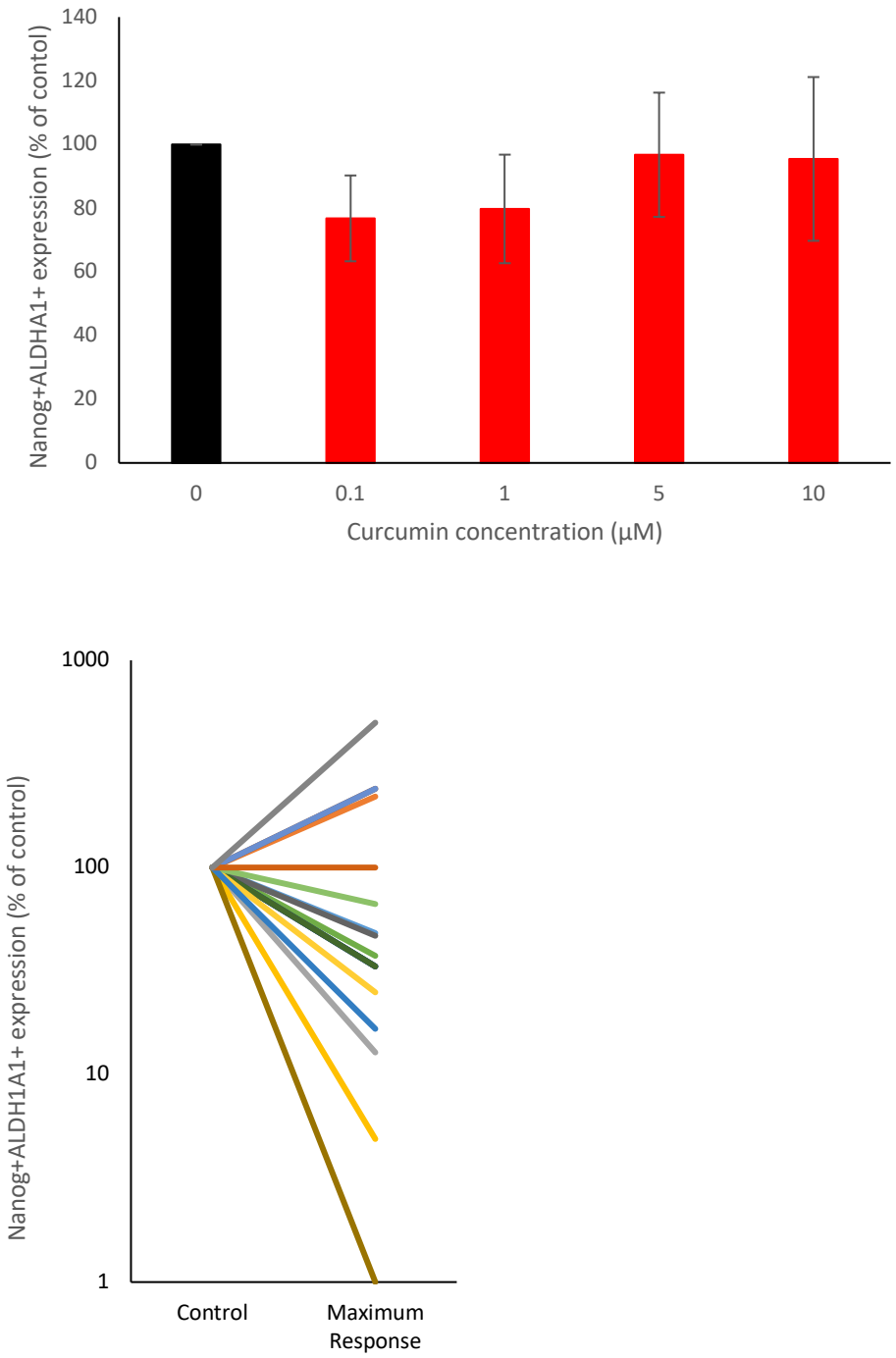


Figure 6-10. Curcumin has no effect on Nanog$^+$ALDH1A1$^+$ expression in primary colorectal tissues. Primary CRC tissues were cubed and treated with curcumin for 24 hours (0-10 µM). Tissues were harvested and single cells obtained. Subsequently, cells were fixed and permeabilised, antibodies added and analysis carried out. The effect of curcumin on Nanog$^+$ALDHA1$^+$ expression (top) and greatest increase or decrease in Nanog$^+$ALDHA1$^+$ expression is shown (below). Results from a single replicate are shown from 20 patient samples.

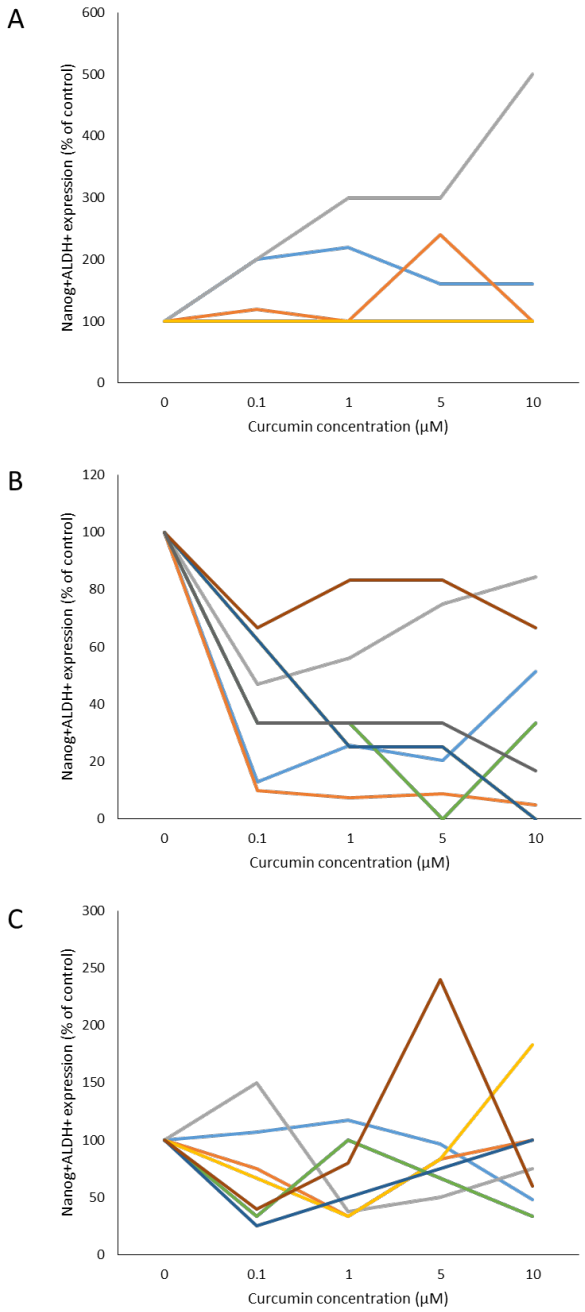


Figure 6-11. Effect of curcumin on Nanog$^+$ALDH1A1$^+$ expression in primary colorectal tissues. Primary CRC tissues were cubed and treated with curcumin for 24 hours. Nanog$^+$ALDH1A1$^+$ expression was assessed. A range of responses were seen. (A) No response (B) Linear treatment response (C) Non-linear response. Results from a single replicate are shown.

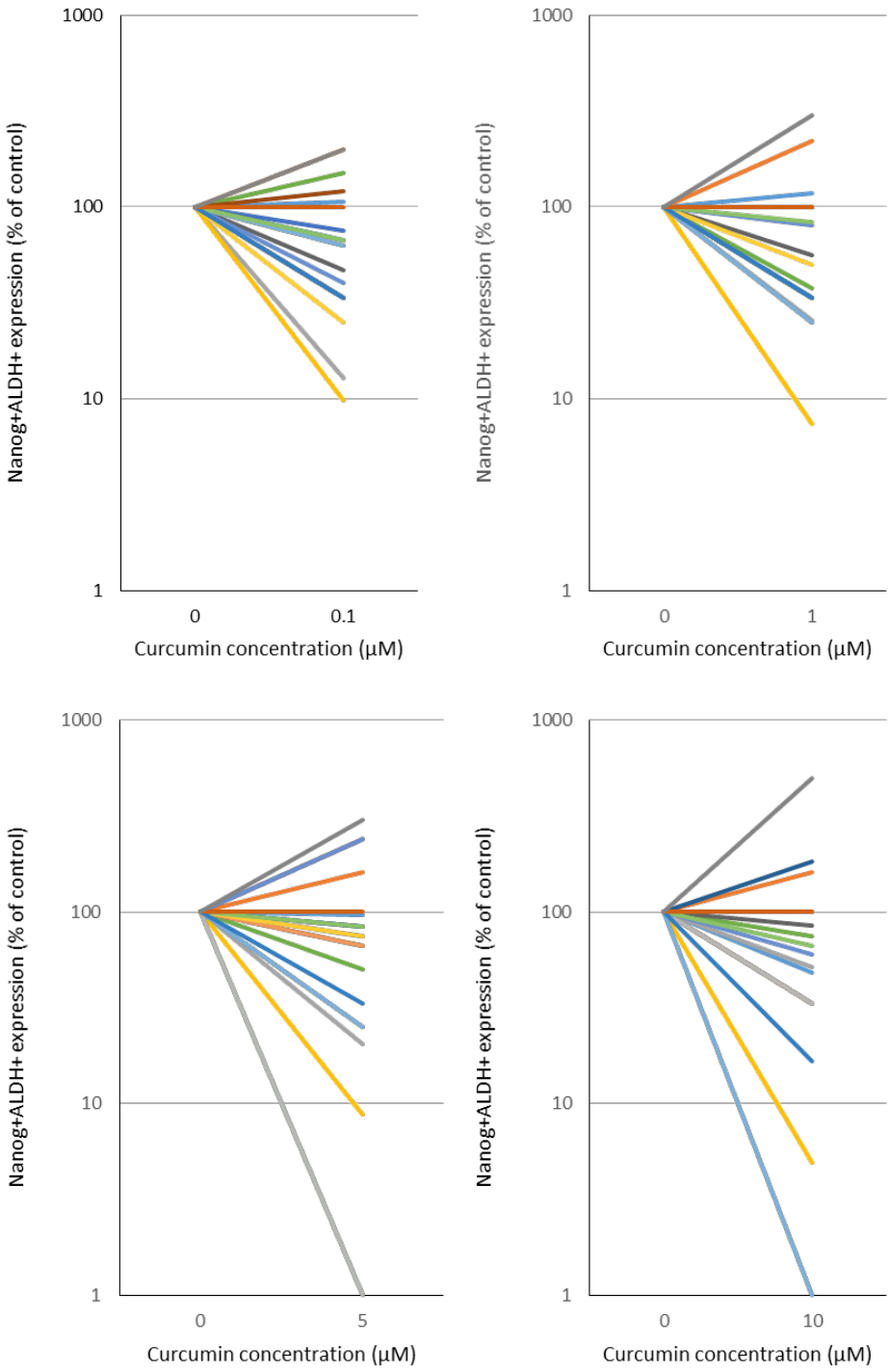


Figure 6-12. Curcumin has no effect on Nanog^{+}ALDH1A1^{+} expression in primary colorectal tissues. Primary CRC tissues were cubed and treated with curcumin for 24 hours (0-10 µM). Tissues were harvested and single cells obtained. Cells were fixed and permeabilised, antibodies added and analysis carried out. Results from a single replicate are shown.

6.8 Conclusion

In this chapter, human CRC and adenoma tissues were used to assess the effects of curcumin *ex vivo*. Methods for *ex vivo* culture have been used for many years. These models have been used in understanding the role of infections as well as the effects of drugs in cancer tissues. A range of tissues have been investigated including lung, colon and prostate tissue [243] [244] [245]. Initially, there were a range of practical difficulties which needed to be overcome. Firstly, pathways to establish tissue procurement were well established within the group. However, the amount of tissue required for these experiments was far higher than routinely received. To carry out explant culture a minimum of 0.5-1 g of tissue was required for a minimum number of wells to assess (x5). This allowed culture of a control and 4 concentrations of curcumin to be assessed by flow cytometry. A small amount of excess tissue was used to create single cells which were stored at -80°C, allowing baseline CSC expression in these tissues to be assessed.

As a result of this limitation, it was unfeasible to carry out experiments in triplicate, which is good laboratory and experimental practice. To obtain larger amounts of tissue required working with the pathology team to help facilitate this. In particular, a there was a focus to obtain adenoma tissues too, allowing premalignant cells to be assessed. When carrying out IHC, even larger pieces of tissue were needed, so that a reasonable amount of tumour tissue could be assessed (taking into account, some of the tissue was stroma, debris and necrotic).

The process to cube tissues was carefully undertaken, this was to ensure that pieces of tissue were big enough to retain microenvironment and stroma, but not so large that treatments were not able to penetrate through the tissues following 24 hours of culture. Lastly, experiments were undertaken to assess the effects of explant culture on CRC tissues. The data here suggests, following 48 hours of *ex vivo* culture, the expression of CSC reduced significantly, compared to baseline. This may be as a result of an increase in the necrotic or non-viable proportion of cells and loss of tissue integrity contributing to debris, as tissue is cultured for approximately 48 hours prior to harvesting for analysis. Though this was not specifically assessed in these experiments, recent published literature from the centre suggests in lung cancer tissues, 24 hours is the optimum culture time of *ex vivo* tissues and levels of serum (0-5%) have no significant implications for tissue viability or integrity. They too also excluded tissues if they were felt to be too necrotic to work with when received. This work also highlights that culture of human

tissues *ex vivo* with agents that are of interest may help to select individuals who are likely to respond to treatment compared to those who may not [242].

The data in this chapter supports the theory that curcumin is able to modulate $Nanog^{+}Ki67^{+}$ expression in human CRC tissues treated *ex vivo*. There are ongoing investigations into the mutational profile of the patient samples which responded compared to those who did not including BRAF mutation and MSI status. In addition, the CMS subtype is being analysed via IHC so that this can also be correlated with response.

7.0 Chapter 7 – Concluding summary

7.1 Evaluating the mechanism of action of curcumin against CRC CSCs

Curcumin is known to target a number of anti-cancer and non-cancer related pathways in malignancies, inflammatory conditions and infections [101]. This multi-targeting mechanism of action, allows it to have a variety of functions and in many ways it works as a combination CRC prevention therapy. Therefore, determining dominant pathways responsible for efficacy remains challenging. For this reason, it was important to consider the effect of curcumin on non-sorted cells, sorted cell populations (such as $ALDH^{high}$ and $ALDH^{low}$) and enriched populations such as that of $HCT116^{GFP/Nanog}$ and $HCT116^{GFP}$ cells. This allowed the effect of curcumin to be investigated in a number of different ways, to try and determine whether CSCs may be a preferential target for curcumin. As the CSC population was relatively small, flow cytometry based assays allowed detection of $Nanog^{+}$ population, and any changes in proliferation and apoptosis to be detected. Gene expression and protein expression changes in Nanog and downstream targets were more challenging to detect robustly, particularly in a non-sorted population.

Notably, much of the cell line data published on curcumin, should be interpreted with caution as often supra-physiological doses are used, or doses which could not be sustained for a prolonged period in clinical populations due to toxicity or concerns regarding compliance [246]. In addition, concentrations detectable in the bloodstream are often far lower than those measured in the colon due to the hydrophobic nature of curcumin. This has implications for the cancer site being investigated. In CRC, as curcumin remains within the colon, it is readily detected, and is clear that the target tissue is being reached [247]. As well as the hydrophobic nature of curcumin clinically, in laboratory experiments, it presents a number of challenges. It is critical that low concentrations of solvents are used in cell culture for treatments, ensuring effects observed are due to the agent being tested [248]. The colour of curcumin can result in false positives in many biophysical and biochemical experiments, particularly those that involve the measurement of fluorescence such as the cignal reporter assay, EMSA assay and flow cytometry. In addition, curcumin is sensitive to light and pH of solvents. During the planning of the experiments presented, careful consideration was given to these challenges of working with curcumin, to ensure robust data was obtained [249, 250].

Another element to consider, particularly with dietary compounds is the phenomenon of non-linear treatment effects possibly resulting in different dosing regimens [251]. There was some evidence from explant data and sphere data, that curcumin was able to induce non-linear effects, with lower doses eliciting a greater response than higher doses. This was more often seen in human patient samples than cell lines. Human samples are more heterogeneous than cell lines and provide an insight into how an intervention may work in a clinical setting. This often also leads to a greater variability in results, compared to cell line data, which can be difficult to extrapolate. A way to overcome this, is to use larger patient numbers, however, this has to be balanced with the feasibility of accessing patient tissues, the cost of processing samples and ethical implications for patients. There is some clinical trial data also supporting this phenomenon such as negative cancer prevention trials to date, and intake of alcohol for example, which has been associated with a J-shaped curve for cardiac prevention. In order to explore the reasons for differences in the dose-response relationships, more in-depth studies would be required using samples from a much larger cohort of patients with different clinical stages of disease and different driver mutations.

An interesting area touched upon, was the possibility of resistance mechanisms and increased cancer risk as a result of cancer prevention agent use. One author has raised this as a key issue for the failures seen with large clinical trials in prevention studies [226]. An experiment was carried out considering this by treating spheres for a period of 2 weeks, replating and allowing the spheres to regrow and treating for a second and third time. The spheres remained sensitive to curcumin treatment, suggesting they were not displaying signs of resistance. This supports the use of curcumin in a way which may involve periods of time taking treatment, and then having a break from treatment and recommencing. This model does not assess the effect of resistance on a non-CSC population. Further work is required in this area.

Extensive work by the group and myself has been carried out assessing the stability of Nanog protein in the presence of curcumin. This line of investigation was considered due to the post translational effects which can modulate Nanog expression including phosphorylation. However, changes in protein stability using emetine and cycloheximide assays, at a range of different time points, in different cell lines (including those enriched for Nanog expression) were not convincingly detected. There is some evidence that aspirin has this effect via Nanog, but there was no evidence of this detected in our hands with curcumin treatment [252]. It may be that Nanog stability is affected via a non-direct pathway, thereby inconsistent changes are seen or no effect is seen. Alternatively,

curcumin is not able to mediate a change in Nanog expression due to changes in protein stability. An alternative explanation is provided by the explant data presented. These data suggests, CSC plasticity and that the proliferating portion of Nanog expressing cells may be potential therapeutic target against a resistant population of cells. CSC plasticity is a novel mechanism, which is being more frequently reported in the literature. If curcumin is able to mediate this effect in the CSC population to decrease the ratio of CSCs to non-CSCs then current therapies in the CRC treatment setting may exhibit greater efficacy. Future explant work may include the assessment of mucin 2, apoptosis and proliferation via flow cytometry, as this would allow confident assessment of cell populations. In addition, it is difficult to assess very small populations via IHC.

In summary, the effects of curcumin were tested in two CSC populations, ALDH and Nanog. This was carried out using cell sorting, and an over expression model. Cell sorting of Nanog, rather than over expression was considered as it was felt to be the most feasible experimental model. In addition, a caveat of these markers, is that they have been used to characterise CRC, but limited work has been carried out in a premalignant setting. The work in this thesis helps to contribute to this. Importantly, to help characterise dominant pathways needed in a CSC to non-CSC switch, I plan to use RNASeq analysis to gain a broader understanding of the effects of curcumin on Nanog related pathways in a 3D spheroid setting. Pathways or markers of interest which are identified can then be verified using protein expression in cell lines. Following this, key candidate markers can be verified in 3D human tissue explant models.

7.2 Immunomodulatory effects of curcumin

Immunotherapy using check point blockade has transformed standard of care of many malignancies including melanoma, renal and lung cancer in an NHS setting [253-255]. In colorectal cancer, immunotherapy approaches have utility in tumours which are MSI-high [58]. It is important to consider the effects of therapeutic prevention agents and their effects on immune modulation. For example, oral intake of aspirin has been associated with a lower risk of colorectal cancer with a lower abundance of immune cells and tumour infiltrating lymphocytes (TILs). This association was stronger with increased dose and duration of aspirin use in those with low levels of TILs but no effect on high TIL score was observed in human patient tissue samples in a prospective cohort. Interestingly, aspirin is able to modulate CSC properties via down regulating Nanog in colorectal cancer (*in vitro* and *in vivo* murine studies) [256]. Notably, one study has assessed the

effect of curcumin on the immune modulation of patients with colorectal cancer. In a randomised study with 40 patients following colorectal cancer resection, 3 g of curcumin or placebo was given in 2 divided doses before meals for 30 days. A similar study with healthy volunteers (30 participants) was concurrently carried out. Patients did not receive any further oncological treatment during this period (e.g. chemotherapy or radiotherapy). Bloods was collected prior to curcumin treatment, at day 10, day 20 and day 30 and PBMCs isolated. T regulatory cells ($CD4^+CD25^+FOXP3^+$) were significantly suppressed following curcumin treatment in both healthy volunteers and patients. No change was seen in the placebo arm for healthy volunteers or patients. There was a concurrent increase in T effector cells ($CD4^+CD25^-FOXP3^-$). Authors suggest curcumin is able to induce plasticity in T regulatory cells which are modulated into T effector cells, specifically Th1 cells. Following 30 days of treatment, levels of FOXP3 mRNA were similar to that of healthy participants [257]. It is helpful to understand the effect of curcumin on the peripheral blood system, however a window study prior to surgery may have allowed assessment of tissues post treatment. This would need to be balanced with a shorter window of treatment e.g. 1-2 weeks vs 30 days. Assessment of TIL score in tissues treated *ex vivo* with curcumin is underway, as well as correlation with CRC subtype.

7.3 Identifying those most likely to respond to curcumin treatment

A pivotal study using curcumin in a CRC prevention setting in patients who have FAP was recently published. A total of 44 participants were recruited and randomised to receive either curcumin 1500 mg BD or placebo for 12 months [258]. At 4 month intervals, a colonoscopy was performed to assess number and size of adenomatous polyps with a recording of images seen during colonscopy. No change in adenoma size or number was detected following intervention. However, there were a number of limitations to this study. Firstly, there was a large variety in the patients recruited, in terms of age, and types of colon e.g. no colectomy, colectomy with anastomosis and anal pouch formation. In addition, there was a higher number of female patients, whilst sporadic CRC is higher in men. Secondly, there was a 3 month wash out period of potential agents which are thought to modulate polyps such as NSAIDs and green tea. This included a wash out period of curcumin and turmeric. It is unclear how many individuals were taking these agents or how long they were taking these in the active and placebo group. This may have a bearing on the results seen, as if individuals were taking these agents for a long time then, the slow effects of these agents were already

apparent and it would be unlikely to observe a change as a result of 12 months treatment. In addition, the polyps present may already be resistant or altered as a result of these agents. Importantly, though statistically not significant, the curcumin arm had more polyps which were larger than the controls, and a greater variability at the start of the trial. In addition, it was not clear how the colonoscopy assessors were blinded to the videos of the colon when assessing polyp size. This is a factor, as in our experience the bowel is stained yellow following curcumin treatment. Lastly, there were no details given to suggest if colouring had or had not been used in the placebo. Overall this was a pragmatic study, however the number of patients involved was small with large variability in the baseline characteristics. In addition, though FAP has traditionally been used to assess the efficacy of chemoprevention agents, the results are not always applicable to cancers in a sporadic CRC setting. It will be helpful to consider the Ras, MSI and BRAF status of these patients, as this may help to identify those who are most amenable to treatment. This is currently ongoing in tissues which were used for our explant studies. This study highlights the numerous challenges faced with trials in a cancer prevention setting.

7.4 Developing Nanog as a druggable target in colorectal cancer

There is a great deal of pharmaceutical interest in targeting CSCs, as demonstrated by table 1-6 in chapter 1. However, there are concerns identifying targets which are specific to CSCs and managing any resulting toxicities. More importantly, due to the CSC plasticity, therapies may need to be considered in combination with a range of treatments so that differentiated cells as well as CSCs are targeted. Nanog is thought to be specific to cancer stem-like cells which makes it a favourable molecule for targeting. The profiling of CRC, adenoma and normal tissues, along with outcomes of patients may give an insight into the prognostic effects of Nanog and CSCs in CRC. There is interest in attempting to target transcription factors rather than pathways. These are often unique proteins to CSCs or have very low expression in somatic cells. Currently, small molecule Nanog inhibitors are not commercially available. A limitation to developing Nanog inhibitors, is the paucity of data on Nanog protein structure. Robust data exists regarding the homeodomain which has been characterised by NMR and crystallography (murine and human), but other domains are yet to be published. Understanding the structure of Nanog and potential binding regions with curcumin may help to develop small molecule inhibitors of Nanog. This is being undertaken by a collaborator. These could be used in the treatment of CRC, potentially in combination with chemotherapy or immunotherapy.

Alternative methods of targeting Nanog include RNAi or using immunological methods e.g. vaccination. Immunomodulation involving Nanog has been considered as a viable option by some authors. It is felt the immune system may be trained to have a ‘memory function’ against CSC transcription factors in those with cancer and healthy individuals (Oct4) [259].

Overall, the data presented in this thesis supports an interaction between curcumin and Nanog in colorectal *CSCs in vitro* and *ex vivo*. Ongoing studies to identify molecular and histological features that delineate responders from non-responders are underway. Nanog may serve as a biomarker in clinical trials to identify individuals most amenable to curcumin alone or in combination for the prevention and/or treatment of CRC. In addition, there is a suggestion curcumin is able to direct CSCs to a non-CSC phenotype as the mechanism of action. I am hoping to investigate this further using RNASeq. The interaction between curcumin and Nanog will be explored further using NMR. This will aid the development of novel Nanog inhibitors which can be used in a combination therapy trials for the treatment of CRC.

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